USER GUIDE

gibco

# Freedom™ CHO-S™ Kit

For transfection of CHO-S™ Cells (cGMP-banked) and development of stable cell lines for protein production

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# About this guide

## Revision history

| Revision | Date | Description |
|---|---|---|
| A.0 | July 2015 | Addition of Dynamis™ fed-batch guidance, plus minor revisions |
| 3.0 | 2013 | Addition of Quick Reference protocols, plus minor revisions |
| 2.0 | 2012 | Addition of Neon™ protocol, plus minor revisions |
| 1.0 | 2011 | New manual |

# Product information

## Product description

**Freedom™ CHO-S™ Kit**

The Gibco™ Freedom™ CHO-S™ Kit (Cat. no. A13696-01), co-developed by Thermo Fisher Scientific Corporation and ProBioGen AG, is designed for easy cloning and expression of recombinant proteins in Chinese hamster ovary (CHO)-derived suspension culture CHO-S™ Cells (cGMP-banked). The Freedom™ CHO-S™ Kit provides reagents for:

- cloning one or two genes that encode your protein(s) of interest
- transfecting the DNA into CHO-S™ Cells (cGMP-banked) with high efficiency
- generating stable cell lines that produce your protein of interest using a single, chemically-defined animal origin-free media

## Kit contents and storage

| Item | Amount | Shipping | Storage |
|---|---|---|---|
| CHO-S™ Cells (cGMP-banked), $1 \times 10^7$ cells/mL | 1 mL | Dry ice | Liquid nitrogen vapor phase |
| CD FortiCHO™ Medium | 1000 mL | Ambient temperature | 2°C to 8°C, protect from light |
| Anti-Clumping Agent | 20 mL | Ambient temperature | 2°C to 8°C |
| OptiPRO™ SFM | 100 mL | Ambient temperature | 2°C to 8°C, protect from light |
| FreeStyle™ MAX Reagent | 1 mL | Ambient temperature | 2°C to 8°C, do not freeze |
| L-glutamine, 200 mM | 100 mL | Dry ice | –5°C to –20°C, protect from light |
| Puromycin, 10 mg/mL | 20 mL | Dry ice | –5°C to –20°C, protect from light |
| One Shot™ TOP10 Chemically Competent *E. coli* (20 reactions) | 21 × 50 µL | Dry ice | –80°C |
| Freedom™ pCHO 1.0 Expression Vector | 1 kit | Dry ice | –5°C to –20°C |
| Flash drive (contains the user manual for the Freedom™ CHO-S™ Kit) | 1 flash drive | Ambient temperature | Room temperature |

## Materials required but not included

Unless otherwise indicated, all materials are available on our website: **www.thermofisher.com/lifescience**.

- Methotrexate (MTX), available from Sigma-Aldrich as methotrexate hydrate (Cat. no. A6770 or M8407)
- Disposables for molecular biology and cell culture (polypropylene conical centrifuge tubes, microcentrifuge tubes, T-flasks, shaker flasks)
- Cell strainer, such as BD Biosciences 40-µm nylon mesh cell strainer (BD Biosciences, Cat. no. 352340)
- Static and shaking incubators with humidity and $CO_2$
- Biosafety cabinet (i.e., laminar flow hood) for all cell manipulations

## Description of the system

**Components of the Freedom™ CHO-S™ Kit**

- **CHO-S™ Cells (cGMP-banked)**: cGMP-banked, CHO-derived cells adapted to high density, serum-free suspension culture in chemically-defined medium that are capable of producing high levels of secreted, recombinant protein. See page 9 for more information.
- **Freedom™ pCHO 1.0 vector**: A plasmid for cloning ORFs containing a mammalian secretion signal and up to two subunits of your protein(s) of interest. See page 60 for the map and features of the vector.
- **CD FortiCHO™ Medium**: Chemically defined, animal-origin-free, serum-free medium formulated for growth of CHO-S™ Cells (cGMP-banked) and for expression of the recombinant protein(s) of interest. See page 10 for more information.
- **FreeStyle™ MAX Reagent**: A proprietary, AOF (animal origin-free) formulation for high transfection efficiency of plasmid DNA into CHO-S™ Cells (cGMP-banked). See page 12 for more information.
- **Additional components**: One Shot™ TOP10 Chemically Competent *E. coli* cells used for cloning your gene(s) into the Freedom™ pCHO 1.0 vector, OptiPRO™ serum-free medium (SFM) for optimal DNA:lipid complex formulation, L-glutamine supplement for increased medium stability, Anti-Clumping Agent to prevent cell clumping, and Puromycin for stable cell line selection.

**Freedom™ pCHO 1.0 vector**

The Freedom™ CHO-S™ Kit contains the Freedom™ pCHO 1.0 vector, designed by ProBioGen AG, to express one or two genes of interest downstream of the vector's two different hybrid CMV promoters. This vector contains the dihydrofolate reductase (DHFR) selection marker and a puromycin resistance gene, allowing selection using MTX and Puromycin simultaneously. See page 60 for more information on the Freedom™ pCHO 1.0 vector.

**IMPORTANT!** Your gene(s) of interest must be engineered with a mammalian secretion signal for secreted protein expression into the cell culture media.

### Advantages of the Freedom™ CHO-S™ Kit

The Freedom™ CHO-S™ Kit provides the following advantages for protein production in mammalian cells:

- cGMP-banked CHO-S™ Cells, derived from CHO cells (Roy et al., 1999; Schifferli, 1999), provide stable and accurate glycosylation and are observed to yield functional glycoproteins (Sheeley et al., 1997; Werner et al., 1998).
- Freedom™ pCHO 1.0 is a single vector containing strong promoter elements designed to ensure high expression levels of one or two subunits.
- A single medium (CD FortiCHO™ Medium) is used for the entire stable cell line development process (cell passage, transfection, selection, and limiting dilution cloning, with easy transition into Dynamis™ production medium for production), which eliminates the need for any adaptation of cells into other media.
- FreeStyle™ MAX Reagent offers high transfection efficiency of suspension CHO cells with low cytotoxicity.
- FreeStyle™ MAX Reagent, OptiPRO™ SFM, and CD FortiCHO™ Medium are AOF (animal origin-free) and serum-free.
- Robust, streamlined cell line development process with step-by-step instructions.

### FAQ eBook

We have also created an interactive FAQ eBook to supplement the Freedom™ CHO-S™ Kit User Guide to highlight critical steps and milestones. We highly recommend that you become familiar with both this User Guide and the eBook **http://life-technologies.uberflip.com/i/330570** prior to initiating the workflow.

# CHO-S™ Cells (cGMP-banked)

### Characteristics of CHO-S™ Cells (cGMP-banked)

The CHO-S™ cell line is a stable aneuploid cell line established from the ovary of an adult Chinese hamster (Puck et al., 1958). The CHO-S™ cells (cGMP-banked) are:

- prepared from low passage Master Cell Bank cultures derived from parental CHO-S™ cells that were re-cloned by limiting dilution in CD CHO Medium, and selected for their superior serum-free cell growth and transfection efficiencies (Ciccarone et al., 1999; Schifferli, 1999).
- adapted to serum-free suspension growth.
- compatible with CD FortiCHO™ Medium for all steps of the workflow.
- compatible with FreeStyle™ MAX Reagent for high transfection efficiency.
- manufactured under cGMP guidelines and banked in CD CHO Medium, a serum-free, protein-free, and chemically defined medium that is formulated with no components of animal or human origin (Gorfien, 1998). Frozen cells are supplied at a concentration of $1 \times 10^7$ cells/mL and can be thawed directly into CD FortiCHO™ Medium (see page 18).
- available for commercial use with purchase of separate documentation package and license.

### Parental cell line

Chinese Hamster Ovary (CHO) cells are among the most commonly used cell lines for transfection, expression and large-scale production of recombinant proteins. The CHO cell line is a stable aneuploid cell line established from the ovary of an adult Chinese hamster (D'Anna, 1996; D'Anna et al., 1997; Deaven & Petersen, 1973; Puck et al., 1958).

**Note:** While cells were banked in CD CHO Medium, cell thaw and all subsequent workflow steps are performed directly in the kit-provided CD FortiCHO™ Medium without any separate adaptation steps.

**CAUTION!** As with other mammalian cell lines, when working with CHO-S™ Cells (cGMP-banked), handle them as potentially biohazardous material under at least Biosafety Level 1 containment.

Note that the CHO-S™ Cells (cGMP-banked) are provided in freezing medium containing DMSO, and the components of the product may be absorbed into the body through the skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Always wear suitable protective clothing and gloves when handling CHO-S™ Cells (cGMP-banked).

# CD FortiCHO™ Medium

## Introduction

CD FortiCHO™ Medium is a chemically defined, animal-origin-free (AOF), serum-free medium for high-density suspension culture of untransfected or transfected CHO-S™ Cells. The medium contains no human or animal origin components.

**IMPORTANT!** Do not use CD FortiCHO™ Medium containing either MTX and/or Puromycin to propagate untransfected CHO-S™ Cells (cGMP-banked). Parental CHO-S™ cells are sensitive to both Puromycin and MTX in the concentrations used below for selection.

Only cells that have both increased DHFR enzyme and an active puromycin resistance gene, such as cells that have been transfected with Freedom™ pCHO 1.0 vector, can be propagated in CD FortiCHO™ Medium + MTX and/or Puromycin. Parental CHO-S™ cells, however, can be propagated in CD FortiCHO™ Medium supplemented with L-glutamine.

If completing the Freedom™ CHO-S™ workflow (from thaw to tertiary screen) in a single medium lot, we recommend that you pre-order or reserve at least 25 L now. See page 63 for ordering information.

## Features of CD FortiCHO™ Medium

CD FortiCHO™ Medium is:

- developed for the growth of Chinese Hamster Ovary (CHO) cells and expression of recombinant proteins in suspension culture.
- made with animal origin-free, chemically defined components and contains no proteins, hydrolysates, or components of unknown composition.
- formulated without L-glutamine for flexibility of use, and to avoid ammonia accumulation (however, it should be supplemented as required).
- formulated to minimize potential lactic acid accumulation under typical culture conditions.
- **Note:** Additional glucose supplementation may be required for terminal batch cultures as determined empirically or by using our suggested simple glucose fed-batch protocol on page 36.

**Prepare complete CD FortiCHO™ Medium**

- CD FortiCHO™ Medium requires supplementation with L-glutamine. Aseptically add L-glutamine to a final concentration of 8 mM to the medium before use.

  **Note:** We recommend that you do not use thawed L-glutamine or medium supplemented with L-glutamine beyond one month. See page 59 for all CD FortiCHO™ Medium formulations used in this kit.

  **IMPORTANT!** 8 mM L-glutamine is used for all steps in this manual except limiting dilution cloning, which requires 6 mM L-glutamine. See page 37 for more details.

  **IMPORTANT!** Anti-Clumping Agent will interfere with FreeStyle™ MAX transfection. Do not use CD FortiCHO™ Medium containing Anti-Clumping Agent during or for the two passages prior to transfection.

- For MTX + Puromycin selection, aseptically add drugs fresh to CD FortiCHO™ Medium. Do not store drug-containing CD FortiCHO™ Medium. See page 31 for details on selection.

- **Store complete CD FortiCHO™ Medium at 2°C –8°C, protected from light.**

**Growth characteristics of CHO-S™ Cells (cGMP-banked) in CD FortiCHO™ Medium**

Typically, CHO-S™ Cells (cGMP-banked) cultured in CD FortiCHO™ Medium have a doubling time in the range of 17–20 hours; however, doubling time can exceed 20 hours during the first few passages after the cells have been thawed.

Do not allow CHO-S™ Cells (cGMP-banked) to reach a cell density above $2 \times 10^6$ cells/mL before transfection, because this may cause a decrease in transfection efficiency.

**Note:** Individual culturing and passaging techniques of CHO-S™ Cells (cGMP-banked) cells may result in experimental variation.

# FreeStyle™ MAX Reagent and OptiPRO™ SFM

### FreeStyle™ MAX Reagent

FreeStyle™ MAX Reagent is a proprietary, animal origin-free formulation for the highly efficient transfection of plasmid DNA into eukaryotic cells. FreeStyle™ MAX Reagent is specifically formulated to achieve the highest transfection efficiencies and lowest cytotoxicity in CHO-S™ Cells (cGMP-banked) and other cell types.

FreeStyle™ MAX Reagent is also available separately; see page 63 for ordering information.

**Store FreeStyle™ MAX Reagent at 2–8°C. Do not freeze.**

### OptiPRO™ SFM

OptiPRO™ SFM is included with the Freedom™ CHO-S™ Kit to facilitate optimal formation of DNA-lipid complexes. OptiPRO™ SFM is a serum-free medium that is devoid of components of animal or human origin. OptiPRO™ SFM has an ultra-low protein concentration of 7.5 µg/mL.

OptiPRO™ SFM is also available separately; see page 63 for ordering information.

**Store OptiPRO™ SFM at 2–8°C, protected from light.**

# Selection reagents

### Puromycin

The Freedom™ pCHO 1.0 vector contains the puromycin resistance gene, which confers resistance to the antibiotic Puromycin. Puromycin is available separately; see page 63 for ordering information.

Puromycin is an aminonucleoside that blocks protein synthesis in mammalian cells by interfering with ribosomal function. Expression in mammalian cells of the bacterial gene *pac*, derived from *Streptomyces alboniger*, results in detoxification of Puromycin (de la Luna & Ortin, 1992; Lacalle et al., 1989; Vara et al., 1985).

**CAUTION!** Puromycin, while non-hazardous, should be handled with care. Avoid contact with skin and eyes. In case of contact with skin or eyes, immediately rinse with water thoroughly and seek medical advice. Wear suitable protective clothing and gloves when handling Puromycin and Puromycin-containing solutions.

### Store Puromycin

Puromycin in liquid form should be stored –5°C to –20°C. Puromycin is stable for one year, if stored properly. Once thawed, the shelf life is only 1 month. We recommend that, upon initial thaw of the 20 mL bottle, Puromycin be aliquoted into smaller volumes (100 µL– 1 mL) for refreezing such that the drug can be thawed as needed during selection. We routinely use 1 mL aliquots and store them at 4°C post-thaw until they are used up or have been thawed for 1 month, whichever comes first.

### Methotrexate (MTX)

Methotrexate (MTX) is a folic acid antagonist that is actively transported into cells by the folate transporter. In the cell, it is converted to a high molecular weight polyglutamate metabolite by folylpolyglutamate synthase, which binds to DHFR and inhibits its activity.

The Freedom™ pCHO 1.0 vector contains the DHFR gene. DHFR catalyzes the reduction of 5, 6-dihydrofolate to 5, 6, 7, 8-tetrahydrofolate, which is essential for DNA synthesis. CHO-S™ Cells (cGMP-banked) have a basal-level of DHFR activity, which is enhanced by transfection with Freedom™ pCHO 1.0 vector. Thus, cells carrying Freedom™ pCHO 1.0 are more resistant to MTX than parental cells.

**CAUTION! CHEMICAL HAZARD.** MTX is toxic to the skin, eyes, and respiratory system. Wear suitable protective clothing, gloves, and eye and face protection when working with MTX. Refer to the product SDS for complete precautions.

### Prepare 1 mM MTX stock solution

**Note:** MTX powder is insoluble in neutral pH aqueous solutions, and the below protocol ensures complete solubilization before its addition to PBS. This volume of MTX is sufficient for both phases of selection. We recommend performing selection using a single lot of MTX stock solution.

1. Dissolve 10 mg MTX in 100–150 µL of 1 M NaOH. Immediately proceed to step 2 as soon as MTX is completely dissolved as prolonged exposure to high pH inactivates MTX.
2. Bring the volume up to 22 mL using PBS.

   The pH of this solution should be 10–11.
3. Filter-sterilize the solution through a 0.22-µm filter.
4. Store the MTX stock solution in 100 µL–1 mL aliquots at –20°C.

### Prepare selection medium

- To prepare selection medium, use complete CD FortiCHO™ Medium supplemented with Anti-Clumping Agent at a 1:100 dilution, and only add the required concentration of Puromycin + MTX fresh each time (see page 59). Do not store media containing Puromycin + MTX.
- Alternatively, Puromycin + MTX can be added to each selection flask at each step of selection.
- Cells will divide once or twice in the presence of lethal doses of Puromycin, so the effects of the drug take several days to become apparent. Complete selection can take 3–7 weeks of growth in selective medium, depending on your transfection, drug stocks, and protein(s) of interest.

**Note:** For a successful selection, Anti-Clumping Agent must be included in the selection medium at a 1:100 dilution. Medium containing Anti-Clumping Agent can be prepared in advance by adding 10 mL of Anti-Clumping Agent per liter of complete medium, or by adding to each flask 100 µL of Anti-Clumping Agent for every 10 mL of culture medium.

Development work with this kit used 10–50 µg/mL of Puromycin, and 100–1,000 nM of MTX. However, because different transfected cells may exhibit different drug sensitivities, we recommend that you generate pools as described in the Experimental Flowchart on page 14 with multiple concentrations of Puromycin + MTX to ensure that you find the optimal conditions for your protein(s).

# Methods

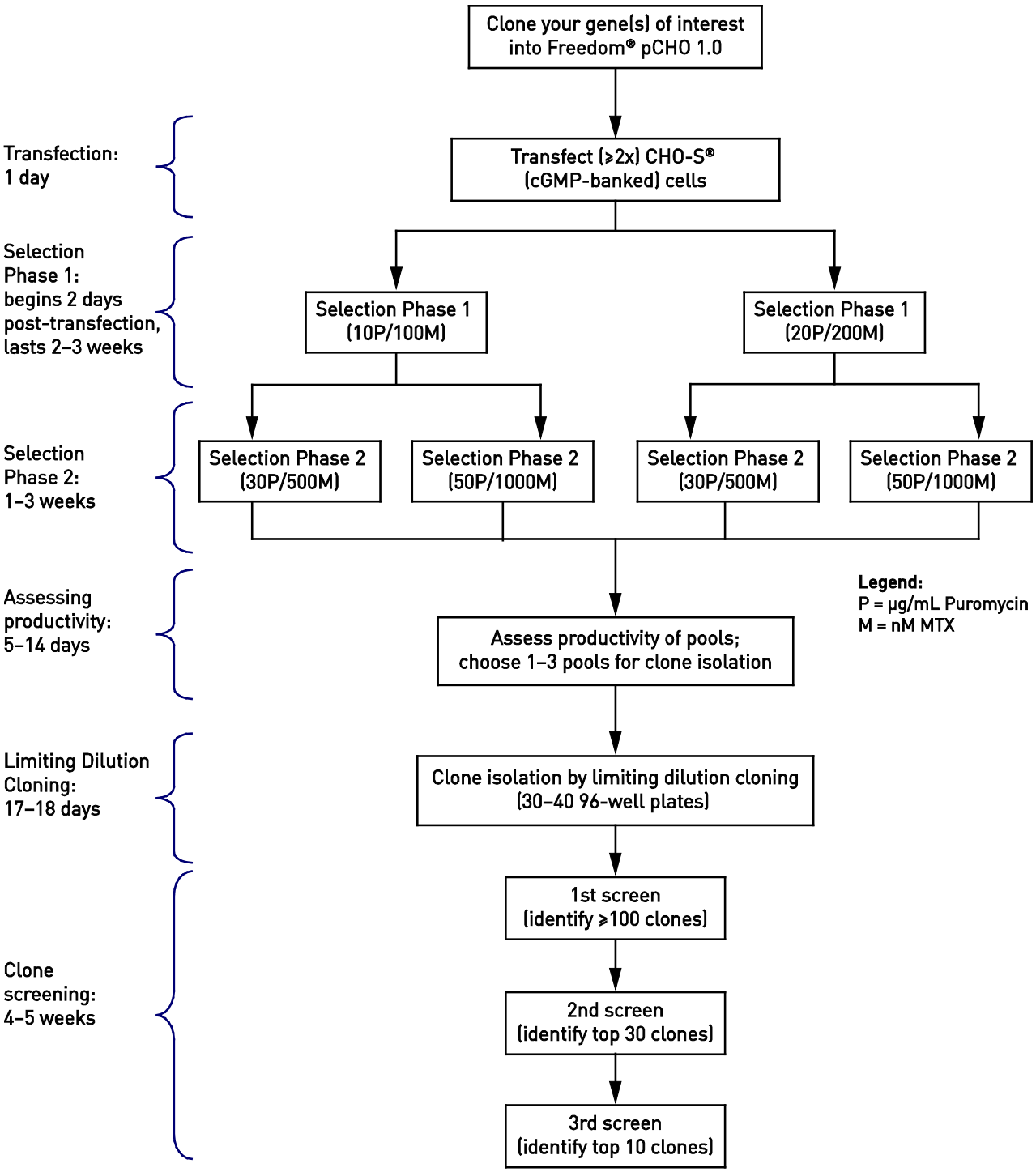
## Experimental flowchart for protein expression

### Introduction

The diagram on the following page schematically depicts the steps necessary to express your one or two-subunit protein(s) of interest using the Freedom™ CHO-S™ Kit, and it shows our recommended experimental path from stable transfectants to clone scale-up.

**Note:** The times shown for various experimental steps are approximations, and the actual times depend on your protein(s) of interest and the specific workflow you choose.

**Note:** For a two-subunit protein, create two expression plasmids, each containing the subunits in a different order (SU1–SU2 or SU2–SU1). Transfect CHO-S™ Cells (cGMP-banked) with each plasmid in parallel at least through transient transfection to identify the optimal gene orientation. For a single-subunit protein, clone your gene of interest behind the first promoter. For information on cloning each subunit of your protein of interest into the Freedom™ pCHO 1.0 vector, refer to page 16. For a map and features of the Freedom™ pCHO 1.0 vector, see page 60.


Clone your gene(s) of interest into Freedom® pCHO 1.0
Transfect (≥2x) CHO-S® (cGMP-banked) cells
Transfection: 1 day
Selection Phase 1: begins 2 days post-transfection, lasts 2–3 weeks
Selection Phase 1 (10P/100M)
Selection Phase 1 (20P/200M)
Selection Phase 2: 1–3 weeks
Selection Phase 2 (30P/500M)
Selection Phase 2 (50P/1000M)
Selection Phase 2 (30P/500M)
Selection Phase 2 (50P/1000M)
Legend:
P = µg/mL Puromycin
M = nM MTX
Assessing productivity: 5–14 days
Assess productivity of pools; choose 1–3 pools for clone isolation
Limiting Dilution Cloning: 17–18 days
Clone isolation by limiting dilution cloning (30–40 96-well plates)
Clone screening: 4–5 weeks
1st screen (identify ≥100 clones)
2nd screen (identify top 30 clones)
3rd screen (identify top 10 clones)

# Create expression plasmids for the Freedom™ CHO-S™ Kit

## Gene optimization

Prior to cloning your gene(s) of interest into the Freedom™ pCHO 1.0 vector, we highly recommend that you have the nucleotide sequences optimized, not only for codon usage, but also for cryptic splice sites, RNA secondary structure, killer motifs, etc. GeneArt™ GeneOptimizer™ software has given us the most consistent success from molecule to molecule, and is a service available from our website (**www.thermofisher.com/lifescience**). You can assess the results of gene optimization of your gene(s) of interest prior to any financial commitments.

## Clone into Freedom™ pCHO 1.0

The Freedom™ CHO-S™ Kit contains the Freedom™ pCHO 1.0 vector. Refer to page 60 for map and the features of the vector.

Using the instructions in this manual, you will:

- For two-subunits, create two expression plasmids by cloning two separate PCR products corresponding to each of the two subunits (SU1 and SU2; SU: subunit) of your protein(s) of choice separately into Freedom™ pCHO 1.0 vector. Because protein expression may depend on the order of the different subunits of your protein(s) of interest, clone SU1 and SU2 in two different combinations using the following insertion sites to optimize protein expression:
  - First insertion site: *AvrII*/*BstZ17I* (blunt)
  - Second insertion site: *EcoRV* (blunt)/*PacI*
  - We recommend testing each linearized expression plasmid in parallel in transient transfection to determine which orientation gives the best protein expression before proceeding with stable pool generation.
- For single-subunit proteins, create a single expression plasmid using the Freedom™ pCHO 1.0 vector and the following insertion site:
  - First insertion site: *AvrII* and/or *BstZ17I* (blunt)

  *Optional:* When creating a single expression plasmid, remove the second expression cassette by *Sfi*I digestion and vector re-ligation.
- For a transfection efficiency control, create a single expression plasmid using a GFP allele inserted into Freedom™ pCHO 1.0 vector at the first insertion site (*AvrII* and/or *BstZ17I* (blunt)). Be sure to prepare the control plasmid using the same reagents and procedures as used for your expression plasmid(s), including the linearization step, before transfecting in parallel to assess transient transfection efficiency.
- For best ligation results, be sure your insert is in molar excess of the plasmid in the ligation reaction. We recommend trying a few insert:vector ratios to ensure the isolation of the desired product (for example, 3:1 and 5:1 insert:vector ratios). You can calculate the amount of insert needed when starting with a known amount of vector as follows:

$$\text{Amount of insert (ng)} = \frac{\text{Size of insert}}{\text{Size of vector}} \times (\text{insert:vector ratio}) \times (\text{ng vector})$$

For example, to achieve a 3:1 insert:vector ratio to ligate a 2 kb insert into 20 ng of 13 kb Freedom™ pCHO 1.0, use 9.2 ng of the insert.

**IMPORTANT!** Your inserts must contain an ATG initiation codon in the context of a Kozak translation initiation sequence for proper initiation of translation in mammalian cells (Kozak, 1987; Kozak, 1990; Kozak, 1991). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position 4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

**(G/A)**NNATG**G**

For the Freedom™ pCHO 1.0 vector, we have successfully used GCCACCATGG.

Also, if you wish to express secreted proteins, be sure to include a signal peptide sequence for the secretion of your protein of interest.

### Freedom™ pCHO 1.0 bacterial selection marker

Freedom™ pCHO 1.0 vector contains the bacterial selection marker *aph*, which allows for selection of transformed cells by generating resistance to the antibiotic kanamycin (Oka et al., 1981; Vakulenko et al., 1987).

- When transforming One Shot™ TOP10 Chemically *E. coli* with the Freedom™ pCHO 1.0 vector, use 50 µg/mL of kanamycin.
- If you wish to use a different strain of *E. coli*, ensure that it is kanamycin-sensitive and conduct a kill-curve study to establish the ideal concentration of kanamycin to use.

### Types of expression plasmids

You will create **two expression plasmids** for two subunits, or create **one expression plasmid** for a single subunit:

| Vector | DNA | Selection |
|---|---|---|
| Freedom™ pCHO 1.0, SU1 upstream of SU2 | mammalian secretion signal and SU1<br>mammalian secretion signal and SU2 | MTX + Puromycin |
| Freedom™ pCHO 1.0, SU2 upstream of SU1 | mammalian secretion signal and SU2<br>mammalian secretion signal and SU1 | |
| Freedom™ pCHO 1.0, single SU | mammalian secretion signal and single SU | |

### Sequence recombinant expression plasmids

We strongly recommend that you analyze your recombinant expression plasmid(s) by sequencing the promoter-gene of interest junctions before transfecting CHO-S™ Cells (cGMP-banked) and expressing your protein(s) of interest. For your convenience, the sequences of the primers used for analyzing the promoter-gene of interest junctions in the recombinant expression plasmid(s) are provided below.

**Note:** The sequencing primers are not supplied as part of the Freedom™ CHO-S™ Kit; they need to be ordered separately and custom synthesized.

| Primer | Primer sequence | Location | Comments |
|---|---|---|---|
| AB-For | 5'-GTCTGAGCCTCCTTGTCTTG-3' | begins ~270 bp upstream of *Avr*II/*Bst*Z17I insertion site | forward primer for EF2/CMV hybrid promoter ORF |
| AB-Rev | 5'-AGAAGACACGGGAGACTTAG-3' | begins ~90 bp downstream of *Avr*II/*Bst*Z17I insertion site | reverse primer for EF2/CMV hybrid promoter ORF |
| EP-For | 5'-GGTGTCGTGAGGAATTTCAG-3' | begins ~285 bp upstream of *Eco*RV/*Pac*I insertion site | forward primer for CMV/EF1 hybrid promoter ORF |
| EP-Rev | 5'-GAGGCAGCCGGATCATAATC-3' | begins ~250 bp downstream of *Eco*RV/*Pac*I insertion site | reverse primer for CMV/EF1 hybrid promoter ORF |

**Recommendation:** The methods for transfection into mammalian cells and the selection process for the Freedom™ CHO-S™ Kit are the same whether you are expressing 1 or 2 subunits. See page 14 for an overview.

## Thaw and subculture CHO-S™ Cells (cGMP-banked)

### Introduction

Follow the protocol below to thaw CHO-S™ Cells (cGMP-banked). The cells are supplied in a vial that contains 1 mL of cells at $1 \times 10^7$ viable cells/mL in 90% complete CD CHO Medium and 10% DMSO. Thaw the cells directly into complete CD FortiCHO™ Medium (supplemented with 8 mM L-glutamine). No separate adaptation is required.

For information on making a research cell bank in CD FortiCHO™ Medium, refer to **Freeze CHO-S™ Cells and make a research cell bank (RCB),** page 20.

### Prepare complete CD FortiCHO™ Medium

- All solutions and equipment that come in contact with the cells must be sterile. Always use proper aseptic technique and work in a biosafety cabinet.
- Supplement CD FortiCHO™ Medium with L glutamine to a final concentration of 8 mM before use. See pages 10 and 59.
- Addition of antibiotics is not recommended.
- CD FortiCHO™ Medium is light sensitive. For optimal results, store medium at 2ºC–8ºC, protected from light.

### Required materials

You need the following reagents and materials before beginning:

- Frozen CHO-S™ Cells (cGMP-banked) (supplied with the kit; store frozen cells in liquid nitrogen until ready to use)
- Complete CD FortiCHO™ Medium (prepared as above; pre-warmed to 37°C)
- 125 mL-polycarbonate, disposable, sterile Erlenmeyer flasks with vented caps
- Orbital shaker set at 130–150 rpm in a 37°C incubator with a humidified atmosphere of 8% $CO_2$

### Shaking speed

The 130–150 rpm shaking speed (as well as all the values used in other places in this manual) is specific to an Infors Multitron 2 shaker incubator with an orbital throw of 25 mm. When using a shaker with a different orbital throw, we recommend that you modify the shaking speed to match the relative centrifugal force ($RCF = 1.118 \times 10^{-5} \times THROW \times SPEED^2$).

### Thawing procedure

1. Remove the cryovial of cells from the liquid nitrogen and thaw quickly (less than 2 minutes) in a 37°C water bath.
2. Decontaminate the outside of the vial with 70% ethanol. Aseptically transfer the entire contents of the cryovial into a disposable, sterile polycarbonate 125 mL-Erlenmeyer shaker flask containing 29 mL of pre-warmed complete CD FortiCHO™ Medium.

   **Note:** Removal of the DMSO from the medium is not necessary.
3. Incubate the cells in a 37°C incubator containing a humidified atmosphere of 8% $CO_2$ in air on an orbital shaker platform rotating at 130–150 rpm.

   **Note:** We have thawed and subcultured CHO-S™ cells at both 130 and 150 rpm with the only difference being a slightly faster growth rate at 150 rpm. The speed range is provided here for your convenience.
4. *Optional*: After 24 hours in culture, sample to determine the cell density and viability using the protocol described below.
5. Once the culture reaches $1 \times 10^6$–$2 \times 10^6$ viable cells/mL, typically 2–3 days post-thaw, expand the culture using the subculturing protocol, (see page 20).

### Determine cell density and viability

Follow the procedure below to determine the viable and total cell counts.

1. Transfer a small aliquot of the cell suspension into a microcentrifuge tube. Be careful to avoid any cell clumps that may be present in the culture.
2. Determine cell viability and cell density using your method of choice.

### Subculture cells

Passage CHO-S™ Cells (cGMP-banked) every 2–3 days into fresh medium. When passaging the cells, use disposable, sterile polycarbonate 125-mL Erlenmeyer shaker flasks with vented caps and pre-warmed complete CD FortiCHO™ Medium (for instructions on preparing complete CD FortiCHO™ Medium, see page 59). We have used a 125-mL shaker flask for 15–40 mL of suspension culture. If larger volumes and/or cell numbers are required, increase the number of 125-ml Erlenmeyer shaker flasks you seed or increase your shaker flask size to allow for increased culture volume.

1. Determine the viable and total cell counts, taking caution to avoid any cell clumps that may be present in the culture.
2. Using the cell density determined in step 1, calculate the volume of cell culture suspension and fresh complete medium needed to seed each new shaker flask by dilution. Seed the culture at a density of $2.0 \times 10^5$ viable cells/mL, if a subculture step is scheduled for 2 days. Seed the culture at a density of $1.0 \times 10^5$ viable cells/mL, if a subculture step is scheduled for 3 days.
3. Transfer the calculated volume of pre-warmed complete CD FortiCHO™ Medium into a sterile 125-mL Erlenmeyer shaker flask.
4. Transfer the calculated volume of cell suspension into the pre-warmed complete CD FortiCHO™ Medium to give a final cell density of $1 \times 10^5$ or $2 \times 10^5$ viable cells/mL, depending on the subculture schedule. Be careful to avoid transferring any cell clumps that may be present in the culture.
5. Incubate the flasks in a 37°C incubator with 70–80% humidified atmosphere of 8% $CO_2$ in air on an orbital shaker platform rotating at 130–150 rpm.
6. Repeat Steps 1–5 as necessary to maintain or expand cells.

It is normal to see a small cell ring form on the wall of the flask and/or cell clumps of ≤1 mm at the bottom of the flask (see image on the right). Make sure not to transfer this or any cell clumps into the next passage flask. You may use a cell strainer to remove cell clumps; however, do **not** use Anti-Clumping Agent prior to transfection.

**Note:** Avoid transferring cell clumps during passaging; this will minimize the likelihood of future clumping.

- Do not allow CHO-S™ Cells (cGMP-banked) to reach a cell density above $2 \times 10^6$ cells /mL transfection to avoid a decrease of transfection efficiency.
- A minimum of 5 passages under $2 \times 10^6$ cells/mL is recommended before proceeding with transfection.
- After 25 passages, thaw a new vial of cells. To maintain sufficient stocks of low-passage cells (i.e., under 25 passages), be sure to freeze aliquots of CHO-S™ cells in liquid nitrogen vapor phase.

## Freeze CHO-S™ Cells and make a research cell bank (RCB)

## Introduction

You may freeze CHO-S™ Cells (cGMP-banked) directly in complete CD FortiCHO™ Medium with 10% DMSO. We recommend that you freeze the cells at a density of ≥1 × $10^7$ viable cells/mL at least 3 passages post-thaw, and that you make a research cell bank of at least 20 vials from the vial of CHO-S™ cells provided in the kit. Guidelines for preparing freezing medium and to freeze cells are provided in this section. These protocols can also be used to freeze cells throughout the cell line development workflow.

## Required materials

- Complete CD FortiCHO™ Medium. See page 11.
- Tissue culture grade DMSO
- Reagents and equipment to determine viable and total cell counts
- Sterile, labeled cryovials
- Sterile, 15-mL or 50-mL conical tubes
- Automated or manual controlled-rate freezing apparatus

## Prepare freezing medium

Prepare freezing medium immediately before use.

1. In a sterile, conical centrifuge tube, mix together the following reagents for every 1 mL of freezing medium needed:

   | | |
   |---|---|
   | Complete CD FortiCHO™ Medium | 0.9 mL |
   | DMSO | 0.1 mL |

   **Note:** We recommend preparing extra freezing medium to compensate for losses during pipetting.

2. Place the tube on ice, or store at 2–8°C until use. Discard any remaining freezing medium after use.

## Freeze cells

The following protocol describes how to prepare 1 mL of $1 \times 10^7$ cells per cryovial. Plan ahead to ensure that you have sufficient cells for the desired number of vials.

1. Grow the desired quantity of CHO-S™ Cells in shaker flasks, harvesting when the cells are in mid-log phase of growth with a viability >90%. Typically the cells will be at ~$1.5 \times 10^6$–$3 \times 10^6$ cells/mL on day of freezing.

   **Note:** Cells should be passaged at least 3 times after thaw before freezing.

2. Transfer the cells to a sterile, conical centrifuge tube.
3. Determine the viable and total cell counts (see page 19) and calculate the volume of the cell suspension and the freezing medium required to yield a final cell density of $1 \times 10^7$ viable cells/mL.
4. Centrifuge the cells at 100–200 × g for 5–10 minutes at room temperature and carefully aspirate the medium.
5. Resuspend the cells in the pre-determined volume of chilled freezing medium (90% complete CD FortiCHO™ Medium and 10% DMSO; see above).
6. Place the cryovials in a microcentrifuge rack and aliquot 1 mL of the cell suspension into each cryovial.
7. Freeze the cells in an automated or manual controlled-rate freezing apparatus following standard procedures. For ideal cryopreservation, the freezing rate should be a 1°C decrease per minute.
8. 24–72 hours after freezing the cells, transfer the frozen vials to liquid nitrogen vapor phase for long-term storage.

   **Note:** You can check the viability and recovery of frozen cells 24 hours after storing the cryovials in liquid nitrogen by following the thawing procedure on page 19.

# Transfect CHO-S™ Cells for stable protein expression using the FreeStyle™ MAX Reagent

## Introduction

Instructions for stably transfecting suspension CHO-S™ Cells (cGMP-banked) using the FreeStyle™ MAX Reagent are provided below. Because individual protein expression may depend on the order of the different subunits of your protein(s) of interest, we recommend performing multiple transfections in parallel using your expression plasmids (see page 17).

## Prepare the plasmid

The Freedom™ pCHO 1.0 plasmid constructs containing your gene(s) of interest must be clean, sterile, and free from contamination with organic solvents and sodium chloride for transfection into CHO-S™ cells. We highly recommend using aseptic techniques and sterile vessels for both the DNA preparation and the restriction enzyme digestion. Chemical contaminants may kill the cells, and salt interferes with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using an endotoxin-free or a low-endotoxin kit such as the PureLink™ HiPure Plasmid Midiprep DNA Kit (see page 64). We have found that the easiest and most efficient way to avoid organic solvents and salts that interfere with transfection is to use the plasmid in the restriction digest mix without any further processing.

## Linearize the plasmid

Prior to using the Freedom™ CHO-S™ Kit to transfect CHO-S™ Cells (cGMP-banked) with your Freedom™ pCHO 1.0 construct, you may linearize the plasmids. Linearizing your vectors may not improve transfection efficiency, but it does increase the chance that the vectors will integrate into the host cell genome without disrupting the gene(s) of interest or other elements required for expression in mammalian cells. Follow the guidelines below to linearize your plasmids.

- We suggest using *Nru I*, which cuts once in the kanamycin resistance gene on the plasmid. Other unique restriction sites are possible. See page 62 for a list of unique sites and non-cutting restriction enzymes. Be sure that your inserts do not contain the restriction enzyme site you use to linearize the vector.

  **Note:** If an appropriate linearization site is not present, you may transfect the circular plasmid.

- After digestion, purification of the plasmid DNA is not required. If purification is desired, be careful to ensure complete removal of all organic solvents and salts. Freedom™ pCHO 1.0 may precipitate during mixing with the FreeStyle™ MAX due to carry-over of organic solvents or salts, and DNA precipitation decreases transfection efficiency.

### Required materials

- CHO-S™ cells cultured in complete CD FortiCHO™ Medium at $1 \times 10^6$ viable cells/mL
- Linearized Freedom™ pCHO 1.0 plasmid DNA containing the subunit(s) of your protein(s) of interest, see page 23.
- FreeStyle™ MAX Reagent (supplied with the kit; store at 4°C until use)
- OptiPRO™ SFM (supplied with the kit; pre-warmed to room temperature)
- Disposable, sterile, 125-mL polycarbonate Erlenmeyer flasks
- Orbital shaker in 37°C incubator with a humidified atmosphere of 8% $CO_2$
- Reagents and equipment to determine viable and total cell counts (e.g., Trypan Blue, hemacytometer, or an automated cell counter)

### Optimal transfection conditions using the FreeStyle™ MAX Reagent

Transfect suspension CHO-S™ cells in 30 mL, with the recommended optimized conditions:

- **Final transfection volume:** 30 mL
- **Number of cells to transfect:** total of $3 \times 10^7$ viable cells (cell density at time of transfection should be $1 \times 10^6$ viable cells/mL)

  **IMPORTANT**: It is best to use CHO-S™ cells that have been passaged at least 5 times without cell densities exceeding $2 \times 10^6$ viable cells/mL.
- **Amount of each plasmid DNA:** 50 µg, regardless of whether the plasmid has been engineered to express 1 or 2 subunits
- **FreeStyle™ MAX Reagent:** 50 µL

**Note:** Further optimization of culture volume or transfection conditions is not necessary for stable cell line production.

### General guidelines for transfection using the FreeStyle™ MAX Reagent

- Calculate the number of CHO-S™ Cells (cGMP-banked) that you will need for your transfection experiment and expand cells accordingly. Make sure that the cells are healthy and greater than 95% viable before proceeding to transfection.
- Prepare high-quality plasmid DNA at a concentration of 1–5 µg/µL in deionized water or TE buffer.
- Carry-over of organic solvents and salts from DNA purification can be toxic to the cells; therefore, DNA purification following linearization is not recommended.
- If possible, use an appropriate GFP plasmid transfected in parallel to determine transfection efficiency.
- Prepare extra volume of cell suspension to ensure that the desired cell number is available at the time of transfection.
- For a one-page quick reference protocol intended to be printed and used in the laboratory, see page 55.

## Transfection procedure using the FreeStyle™ MAX Reagent

Follow the procedure below to transfect CHO-S™ Cells (cGMP-banked) in a 30 mL volume. Handle cells at all steps using aseptic technique in a biosafety cabinet. We recommend including negative controls (no FreeStyle™ MAX Reagent, no DNA) in your experiment to help you evaluate your results. We suggest that you perform at least 2 transfections with each plasmid construct to increase the likelihood of generating the highest-producing stable pools before initiating limiting dilution cloning. Note that the same transfection protocol is used whether you are expressing one or two subunits.

**Note:** For a one-page quick reference protocol intended to be printed and used in the laboratory, see page 55.

**IMPORTANT!** Anti-Clumping Agent will interfere with FreeStyle™ MAX transfection. We have specifically not included Anti-Clumping Agent in any cell passage steps prior to transfection for this reason. If you choose to use Anti-Clumping Agent in your passage medium, be sure to remove it at least 2 passages prior to transfection with the FreeStyle™ MAX Reagent.

1. At 24 hours before transfection, pass CHO-S™ Cells (cGMP-banked) at $5 \times 10^5$ to $6 \times 10^5$ viable cells/mL in complete CD FortiCHO™ Medium. Place the flask(s) on an orbital shaker platform rotating at 130–150 rpm at 37°C, 8% $CO_2$.
2. On the day of transfection, perform a viable cell count. To ensure optimal transfection results, viability of cells must be over 95%.

   **Note:** Cell clumping reduces transfection efficiency. If cell clumping is observed in the passage flask on the day of transfection, we recommend using a cell strainer such as the BD Biosciences 40-µm nylon mesh cell strainer (BD Biosciences, Cat. no. 352340) to obtain a uniform single-cell suspension. Determine the cell count and viability after using the cell strainer.
3. For each transfection or control, seed a new 125-mL flask with $1\times 10^6$ viable cells/mL in 30 mL of pre-warmed, complete CD FortiCHO™ Medium. Place the flask in shaker until ready to transfect.

   **Note:** Do not centrifuge cells prior to transfection, because centrifugation decreases transfection efficiency.
4. Gently invert the tube of FreeStyle™ MAX Reagent several times to mix. Do not vortex.
5. Using a vessel large enough for proper mixing of the final transfection mixture (e.g., 50 mL conical centrifuge tube), add 50 µg of plasmid DNA to a final volume of 1.5 mL of OptiPRO™ SFM, and mix gently.

   **Note:** While the volume of DNA added is not critical, we typically use DNA that is ~1 µg/µL after linearization with *NruI* for transfection, which equates to adding 50 µL of DNA to 1.45 mL of OptiPRO™ SFM.
6. Add 50 µL of FreeStyle™ MAX Reagent into 1.45 mL of OptiPRO™ SFM for a 1.5 mL final volume, and mix gently.

   **Note:** Do not vortex FreeStyle™ MAX stock solution or the FreeStyle™ MAX reagent diluted in OptiPRO™ SFM.

**IMPORTANT!** It is critical that step 7, below, is followed exactly as described in the protocol to achieve the maximum transfection efficiency. Read the remaining steps of the transfection procedure carefully before performing them exactly as described.

7. Immediately add the diluted FreeStyle™ MAX Reagent solution to the diluted DNA solution by first immersing the tip and expelling the solution slowly while swirling the tip.
8. Mix the DNA-FreeStyle™ MAX mixture gently by swirling the tube and incubate the mixture for 10 minutes at room temperature to allow DNA-lipid complexes to form. Do not incubate the mixture for longer than 20 minutes.

   **Note:** If precipitation is observed upon addition of FreeStyle™ MAX Reagent to DNA, do not proceed with transfection. Instead, prepare fresh dilutions of your DNA and FreeStyle™ MAX reagent, and ensure that your tip is immersed in the DNA solution when adding the diluted FreeStyle™ MAX Reagent.
9. Drop-wise add the 3 mL of DNA:FreeStyle™ MAX Reagent complex into the 125-mL flask containing cells while slowly swirling the flask.
10. Incubate the transfected cell cultures at 37°C, 8% $CO_2$ on an orbital shaker platform rotating at 130–150 rpm.
11. 48 hours after transfection, proceed directly to **Select stable transfectants for protein expression**, page 31.

# *Optional*: Transfect CHO-S™ Cells for stable protein expression using the Neon™ Transfection System

## Introduction

Make sure that the cells are healthy and ≥95% viable before proceeding to transfection. Before starting, calculate the number of CHO-S™ Cells (cGMP-banked) that you will need for your transfections and expand the cells accordingly.

## Required materials

- Suspension CHO-S™ cells cultured in complete CD FortiCHO™ Medium (see page 11 for recipe).
- Linearized Freedom™ pCHO 1.0 plasmid DNA containing the subunit(s) of your protein(s) of interest, prepared as explained on page 23.
- Neon™ Transfection System (see page 64 for ordering information)

  **Note:** Resuspension Buffers R and E2 used in this protocol are Animal-Origin Free (AOF); contact Technical Support (see page 68) if documentation is required.
- Disposable, sterile T-75 and T-25 tissue culture flasks
- Disposable, sterile, 125-mL polycarbonate Erlenmeyer flasks
- Static culture incubator at 37°C with a humidified atmosphere of 8% $CO_2$
- Orbital shaker in incubator or a shaker incubator, set at 37°C with a humidified atmosphere of 8% $CO_2$
- Reagents and equipment to determine viable and total cell counts (e.g., Trypan Blue, hemacytometer, or an automated cell counter)

## Optimal Neon™ transfection conditions

Single Neon™ transfections using the 100-µL tip generate enough cells for protocol optimization. However, because of the scale limitations of the Neon™ Tips, four repeat transfections of each plasmid are required to generate sufficient cells for the subsequent selection steps. The four repeats can be pooled on the day of transfection, or on the day of selection. Our suggested methods for this process are detailed below.

- **Number of cells to harvest per plasmid:** $4.0 \times 10^7$ viable cells (this amount allows for cell loss during washing)
- **Amount of plasmid DNA per repeat:** 48 µg
- **Neon™ electroporation settings:**

  –Pulse voltage: 1500–1600 V

  –Pulse width: 10 ms

  –Pulse number: 2–3 pulses

**Note:** We have observed equivalent transfection efficiency (40–60%) with Freedom™ pCHO 1.0 at 24 hours and 48 hours post-transfection using the above Neon™ settings; the resulting stable pools produce similarly to those generated by FreeStyle™ MAX transfection. We suggest using these settings as a starting point.

If desired, you can further optimize the instrument settings by transfecting cells with the Freedom™ pCHO 1.0 vector expressing GFP and monitoring the transfection efficiency (% of green cells) at 24–48 hours post-transfection.

### General guidelines for Neon™ transfections

- Use only high-quality, linearized Freedom™ pCHO 1.0 plasmid DNA containing the subunit(s) of your protein(s) of interest, prepared as described on page 23.
- Do not purify the plasmid DNA following linearization because carry-over of organic solvents and salts from DNA purification can be toxic to the cells.
- To ensure that the amount of plasmid DNA does not exceed 10% of the total volume used for transfection, the concentration of the plasmid DNA should be at least 1 µg/µL. If possible, perform a control transfection in parallel using an appropriate GFP plasmid to determine the transfection efficiency.
- Discard the Neon™ Tips after two uses and Neon™ Tubes after ten uses.
- Change the Neon™ Tip, Neon™ Tube, and buffer when switching to a different plasmid DNA or cell type.
- Prepare extra cell suspension during passage to ensure that the desired cell number is available at the time of transfection.

  **Note:** The calculations provided include extra cells to allow for loss during washes and to provide sufficient volume of cells and DNA for the Neon™ tip to be filled completely without introducing air into the tip.
- For a one-page quick reference protocol intended to be printed and used in the laboratory, see page 56.

### Prepare cells for Neon™ transfection

1. 24 hours before transfection, passage CHO-S™ cells at $1 \times 10^6$ viable cells/mL in complete CD FortiCHO™ Medium. Place the flask(s) on an orbital shaker platform rotating at 130–150 rpm at 37°C, 80% relative humidity, and 8% $CO_2$.

   **IMPORTANT!** Cell clumping reduces transfection efficiency. If cell clumping is observed in the passage flask on the day of transfection, we recommend using a cell strainer such as the BD Biosciences 40-µm nylon mesh cell strainer (BD Biosciences, Cat. no. 352340) to obtain a uniform single-cell suspension. Determine the cell count and viability **after** using the cell strainer.
2. On the day of transfection, count the cells and harvest the required amount of cells by centrifugation at 100–200 × g for 5–10 minutes.

   To ensure you have sufficient cells, harvest $4.0 \times 10^7$ viable cells per plasmid to be transfected ($1.0 \times 10^7$ viable cells per repeat × 4 repeats per plasmid).
3. Wash the cells two times with at least 20 mL of phosphate-buffered saline (PBS) without $Ca^{2+}$ and $Mg^{2+}$. For accuracy, recount the cells prior to centrifugation in the second PBS wash.

   **Note:** During the wash steps, prepare the Neon™ system and Neon™ Tube, see page 29.
4. Resuspend the cell pellet in an appropriate volume of Resuspension Buffer R (included with Neon™ Kits) to a final density of $5.0 \times 10^7$ cells/mL based on the cell count performed in step 3. For example, if $4.0 \times 10^7$ viable cells remain after the wash in step 3, then you will need to resuspend the cells in 0.8 mL of Resuspension Buffer R to achieve the desired $5.0 \times 10^7$ viable cells/mL.

**IMPORTANT!** Because the cells in Resuspension Buffer R need to be transfected within 10 minutes of resuspension, we recommend only resuspending enough cells for 6 repeats at a time. If more than 6 repeats are desired, we suggest centrifuging the total desired number of cells in the first PBS wash step, but only performing the second PBS wash and step 4 using a maximum of $5.0 \times 10^7$ viable cells at a time.

5. Prepare two T-75 flasks for each plasmid to be transfected by filling the flask(s) with 10 mL of complete CD FortiCHO™ Medium without antibiotics; place flask(s) in a humidified incubator set at 37°C and 8% $CO_2$ while the cells are being prepared for transfection.

### Neon™ transfection procedure

1. Fill the Neon™ Tube with 3 mL of Electrolytic Buffer E2.

   **Note:** Change the Neon™ Tube after ten uses. Use a new Neon™ Tube for each new plasmid DNA or cell type being transfected.

2. Insert the Neon™ Tube in the Neon™ Pipette Station until you hear a click.

3. Transfer the plasmid DNA (12 µg DNA per repeat) into a sterile, 1.5-mL microcentrifuge tube.

4. Taking care to not introduce air bubbles during pipetting, add 120 µL of cells per repeat to the tube containing the plasmid DNA and gently mix. Because of the configuration of the Neon™ Tip, only 100 µL of cells-DNA mixture will be transferred to the tip. As such, approximately $5 \times 10^6$ cells will be transfected per repeat.

   Alternatively, for each plasmid to be transfected, create a 4X mixture by mixing 48 µg DNA and 480 µL cells. Be sure to carefully mix the cell-DNA suspension each time before drawing it up into the Neon™ Tip.

5. Press the push-button on the Neon™ Pipette to the second stop to open the clamp and insert the top-head of the Neon™ Pipette into the Neon™ 100-µL Tip until the clamp fully picks up the mounting stem of the piston. Gently release the push-button, continuing to apply a downward pressure on the pipette, ensuring that the tip is sealed onto the pipette without any gaps.

   **Note:** Change the Neon™ Tip after two uses. Use a new Neon™ Tip for each new plasmid DNA or cell type being transfected.

6. Press the push-button on the Neon™ Pipette to the first stop and immerse the Neon™ Tip into the cell-DNA mixture. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon™ Tip.

**IMPORTANT!** Ensure that there are absolutely NO air bubbles in the Neon™ Tip before proceeding. It may take a few attempts to properly fill the Neon™ Tip.

7. Insert the Neon™ Pipette with the sample vertically into the Neon™ Tube placed in the Neon™ Pipette Station until you hear a click.
8. Ensure that you have the desired instrument settings (see page 27), and then press Start on the touchscreen.

   The Neon™ device automatically checks for the proper insertion of the Neon™ Tube and Neon™ Pipette before delivering the electric pulse. The touchscreen displays "Complete" to indicate that electroporation is complete.
9. Remove the Neon™ Pipette from the Neon™ Pipette Station and immediately transfer the sample from the Neon™ Tip by pressing the push-button on the pipette to the first stop into the prepared T-75 flask containing pre-warmed medium without antibiotics. You can use the same Neon™ Tip and T-75 flask for a second repeat before discarding the tip into an appropriate biological hazardous waste container.
10. Perform steps 5–9 for the remaining repeats, seeding duplicate repeats from the same Neon™ Tip into a single T-75 flask.
11. Gently rock the T-75 flasks for even distribution of the cells. Incubate the flasks at 37°C in a humidified static incubator maintained at 8% $CO_2$.
12. 48 hours after transfection, pool duplicate flasks for each plasmid transfected and proceed directly to **Select stable transfectants for protein expression**, page 31.

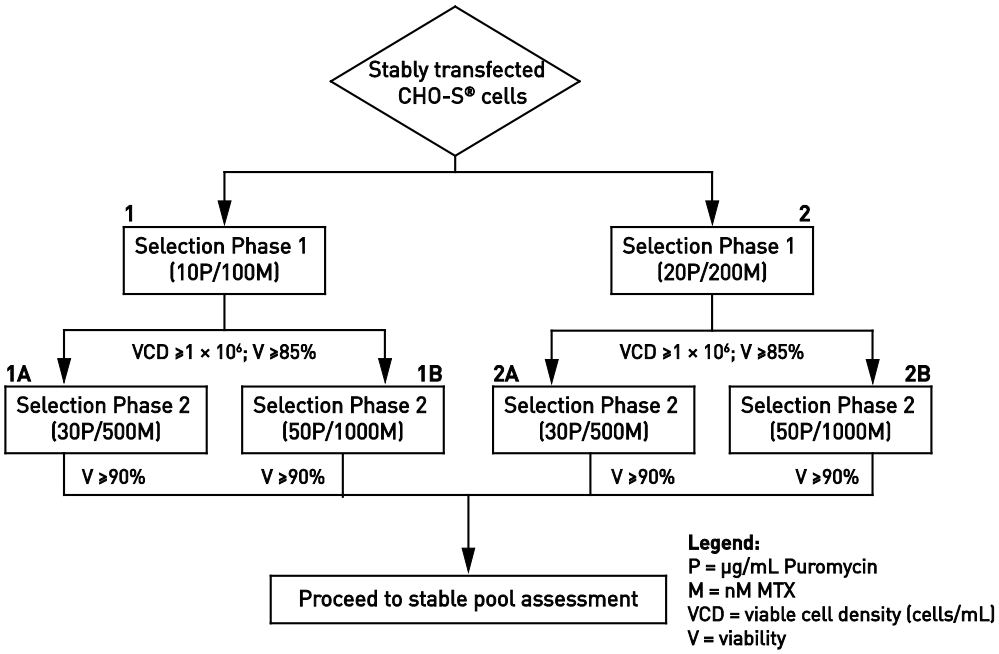
# Select stable transfectants for protein expression

## Introduction

To obtain cell lines that produce high levels of your protein(s), a two-phase selection scheme generates up to 4 pools of stably-transfected cells in which the linearized Freedom™ pCHO 1.0 has integrated into the host cell genome. Perform the selection using complete CD FortiCHO™ Medium containing a combination of Puromycin and MTX. Note that only cells that have been transfected with the Freedom™ pCHO 1.0 construct can be propagated in CD FortiCHO™ Medium containing Puromycin and MTX, because untransfected CHO-S™ Cells (cGMP-banked) lack *pac* activity and only have basal *DHFR* activity.

## Workflow

The following flowchart depicts the major steps of the two-phase selection scheme to generate a pool of stably-transfected cells. The two-phase selection scheme takes approximately 3–7 weeks to complete.




## Required materials

- T-flasks (T-150, T-75)
- Shaker flasks
- MTX (available from Sigma, Cat. no. A6770 or M8407)
- Anti-Clumping Agent (Cat. no. 0010057AE)
- Static and shaking incubators.

## Puromycin and MTX

The Freedom™ pCHO 1.0 vector contains the puromycin resistance gene, which confers resistance to the antibiotic Puromycin, and DHFR, which can be selected for by the addition of MTX.

Complete selection can take up to seven weeks of growth in selective medium.

### Prepare selection medium

- To prepare complete CD FortiCHO™ Medium containing Puromycin and MTX, use complete CD FortiCHO™ Medium supplemented with Anti-Clumping Agent at a 1:100 dilution, and only add the required concentration of Puromycin and MTX fresh each time. Do not store media containing Puromycin and MTX.
- Alternatively, Puromycin and MTX can be added fresh to each selection flask during cell passage.

  **Note:** Development work with this kit used 10–50 µg/mL of Puromycin, and 100–1,000 nM of MTX. However, because different transfected cells may exhibit different drug sensitivities, we recommend that you generate pools with different concentrations of Puromycin + MTX to ensure that you have at least one set of conditions that works with your protein(s) (see Experimental Flowchart, page 14).

  **Note:** For the success of selection, it is critical that Anti-Clumping Agent (Cat. no. 0010057AE, see page 64) be included at a 1:100 dilution in complete CD FortiCHO™ Medium. Medium containing Anti-Clumping Agent can be prepared in advance by adding 10 mL of Anti-Clumping Agent per liter of complete medium, or by adding 300 µL of Anti-Clumping Agent to each 30 mL culture flask.

### Select stable transfectants for protein expression

- The detailed protocols on pages 33–35 describe a two-phase selection scheme that typically takes 3–7 weeks to generate stable pools. Because each phase of selection uses two different selection pressures, each transfection yields 4 stable pools.
- For detailed workflow diagrams of the two-phase selection scheme, intended to be printed and used in the laboratory, see pages 57–58.
- 48 hours after transfection, passage transfected CHO-S™ cells in selection medium (complete CD FortiCHO™ Medium, 1:100 dilution of Anti-Clumping Agent and varying amounts of Puromycin and MTX) as described in the protocols below (pages 33–35).

  **Note:** While Anti-Clumping Agent-containing complete CD FortiCHO™ Medium can be prepared in advance, we recommend that media containing Puromycin and MTX only be prepared fresh for each passage.

### Selection Phase 1 (2 – 3 weeks)

Initiate the first phase of selection 48 hours post-transfection following the protocol below.

*Highly recommended*: Remove a sample from each transfection to determine the transient levels of your protein(s) of interest using an appropriate quantitation assay. This will confirm that transfection was successful, and will provide a useful point of reference when characterizing stable cell pools following selection.

Prepare media containing Puromycin and MTX fresh each time. Do not re-use prepared Puromycin and MTX media. Puromycin and MTX can be added to each flask individually, or, when multiple flasks require the same selection pressure, to a larger volume of medium to be used to seed multiple flasks.

For a one-page workflow diagram of Phase 1 selection, intended to be printed and used in the laboratory, see page 57.

1. Pre-warm selection medium (complete CD FortiCHO™ Medium supplemented with Anti-Clumping Agent at 1:100 dilution) to 37°C.
2. *Optional*: If clumping is observed in the transfection flask, use a cell strainer to obtain a uniform single-cell suspension (we recommend using BD Biosciences' 40-µm nylon mesh cell strainer, Cat. no. 352340).
3. For each transfection, determine viable and total cell counts (see page 19).
4. For each transfection, perform a complete media exchange (by centrifuging the cells and resuspending them in selection medium) and seed two 150-$cm^2$ T-flasks (T 150) at $5 \times 10^5$ viable cells/mL in 40 mL of selection medium per T-flask. If there are insufficient cells, equally reduce the volume of both flasks as needed.
5. To the first T-150 flask containing the transfected cells (1), add Puromycin to a final concentration of 10 µg/mL and MTX to 100 nM (10P/100M). To the second T-150 flask (2), add Puromycin to a final concentration of 20 µg/mL and MTX to 200 nM (20P/200M).

   **IMPORTANT!:** Puromycin can form a white powder at the bottom of the tube at thaw. Ensure that the drug is in solution by inverting the tube several times before opening.
6. Incubate T-flasks at 37 °C, 70–80% relative humidity, and 5–8% $CO_2$ in a static incubator (non-shaking).
7. Sample flasks on day 7 post-selection for a viable cell count only. This will serve as a reference data point; if selection is occurring, you can expect a drop in viability and cell density relative to day 0 of selection. If viability is >30%, proceed directly to step 10. Otherwise, return the flask to incubator and proceed to step 8.
8. Sample flasks on day 10 or 11 post-selection for a viable cell count.

   **Note:** If cell viability is still below the 7 day measured value, perform a complete media exchange by centrifuging the cells and resuspending them in fresh selection medium, adding Puromycin and MTX to maintain the selection pressure. Reduce the volume and T-flask size as needed to keep the viable cell density above $3 \times 10^5$ viable cells/mL.

**Note:** If viability has increased since day 7, perform a complete media exchange as described, and transfer cells to shake flask as described in step 10. While it is typical that cells are >30% viable when this transition occurs, for best results the move to shake flasks should occur as soon as an increase in viability is detected (see step 10).

9. Sample the flasks every 3–4 days and perform a complete medium exchange once a week until the cells show signs of recovery.
10. As soon as the cells show signs of recovery (i.e., increased viability and cell growth from the last measured values), transfer them to a shake flask (SF) at a seeding density of $3 \times 10^5$ viable cells/mL.
11. Incubate the cells on a shaking platform at 37°C, 70–80% relative humidity, 8% $CO_2$, and 150 rpm.

    **Note:** While all steps preceding selection, including pre-culture and transfection of CHO-S™ cells, have used 130–150 rpm (see **Shaking speed**, page 19, we have found more consistent results in selection using 150 rpm. Whatever rpm setting is used during selection, we recommend keeping that setting for all subsequent shake flask pool and clone handling steps, including productivity assessments.
12. Passage the cells in shake flasks every 3–4 days, seeding them at $3 \times 10^5$ viable cells/mL at each passage. Maintain selective pressure by adding Puromycin and MTX appropriate for the volume of fresh media added. Centrifugation for full media exchange is only required when the dilution factor is <2.

    **Note**: We recommend using a cell strainer whenever clumping is observed.
13. Selection Phase 1 is complete when viability exceeds 85% and the viable cell density exceeds $1 \times 10^6$ viable cells/mL.
14. Cryopreserve at least three vials of cells from each Selection Phase 1 pool as a back-up (see page 20) and proceed directly to Selection Phase 2 (page 35).
15. With cells remaining after cryopreservation and seeding Phase 2 selection, seed a separate flask at $3 \times 10^5$ viable cells/mL, but without adding selection pressure, to assess productivity of your Phase 1 pools at day 5 post-seeding. This can be compared back to transient transfection titers to confirm a titer increase, and will serve as a reference for determining if there is an increase in production in your Phase 2 stable pools.

    **Note:** Do not proceed with cultures that have not recovered by day 25 of Selection Phase 1.

    **Note:** During the selection round, cell viability may drop dramatically (to <10%) due to the death of untransfected and transiently-transfected cells. To promote optimal growth of stably transfected cells, maintain cultures as described in the protocol above.

### Selection Phase 2 (1– 3 weeks)

For a one-page workflow diagram of Phase 2 selection, intended to be printed and used in the laboratory, see page 58.

1. For each Selection Phase 1 pool, determine the viable and total cell counts (see page 19).
2. Seed two new 125-mL shaker flasks per Selection Phase 1 pool at $4 \times 10^5$ viable cells/mL in 30 mL of selection medium by performing a complete medium exchange and resuspending cells in fresh medium and then add the appropriate volume of Puromycin and MTX.
3. To the first shake flask (SF-A), add Puromycin to a final concentration of 30 µg/mL and MTX to 500 nM (30P/500M). To the second shake flask (SF-B), add Puromycin to a final concentration of 50 µg/mL and MTX to 1,000 nM (50P/1,000M).

   **Note:** This protocol generates four Selection Phase 2 shake flasks per transfection: 1A, 1B, 2A, and 2B.
4. Incubate the cells on a shaking platform at 37°C, 80% relative humidity, 8% $CO_2$, and 150 rpm. Sample the flasks every 3–4 days; if cells do not show signs of recovery (i.e., cell densities above the last measured value), leave as-is and perform a complete medium exchange once a week. Once cells show signs of recovery, proceed to step 5.
5. Passage the cells in shake flasks every 3–4 days, seeding them at $3 \times 10^5$ viable cells/mL at each passage. Maintain selective pressure by adding Puromycin and MTX appropriate for the volume of fresh media added. Cell pelleting and full media exchange is only required when the dilution factor is <2.

   **Note:** We recommend using a cell strainer whenever clumping is observed.
6. Selection is complete when viability meets or exceeds 90%.

   **Note**: Do not proceed with cultures that have not recovered by day 25 of Selection Phase 2.
7. Cryopreserve at least five vials of cells from each stable pool as a back-up (see page 20) and proceed to
8. 
9. Assess productivity, page 36.

   **Note:** Maintain your stable pools by passaging the cells under final selection pressure conditions until the pool(s) chosen for cloning are determined by assessing pool productivity.

# Assess productivity

## Protein production

To check for production of your protein(s) during stable cell establishment, you can take an aliquot of spent growth medium and perform SDS-PAGE, protein-specific ELISA, or the bioactivity assay of choice to determine that your cells are producing your protein(s) of interest.

**IMPORTANT!** When you have a pool of stably-transfected cells, freeze several aliquots of the pool using the procedure on page 20.

## Assess productivity

The following protocol can be used to assess the productivity in a simple fed-batch culture. Additional nutrition feeds can be added, if desired.

1. Seed fully recovered cell pools (viability >90%) at $3 \times 10^5$ viable cells/mL using 30 mL fresh medium (complete CD FortiCHO™ Medium supplemented with Anti-Clumping Agent at 1:100 dilution) in 125-mL shaker flasks. Inclusion of Puromycin and MTX is not recommended during productivity assessment. Scale up the culture volume appropriately if you increase the sampling volume or frequency.
2. Incubate the cells on a shaking platform at 37°C, 80% relative humidity, 8% $CO_2$, and 150 rpm.

   **Note:** Be sure to use the same rpm speed used during selection.
3. Sample cultures daily or at regular intervals (e.g., on day 0, 3, 5, 7, 10, 12, and 14) to determine the cell density, viability, and productivity until culture viability drops below 50% or day 14 of culture is reached.
4. After sampling, feed the cultures with glucose as follows:
   - Day 3: add 4 g/L of glucose
   - Day 5: add 4 g/L of glucose
   - Day 7: add 6 g/L of glucose

   **Note:** To avoid prolonged culture of pools, we recommend initiating limiting dilution cloning from the pools frozen at the end of Selection Phase 2 (see page 35) instead of stable pool cell trains maintained during the 14-day productivity assessment.

Alternatively, productivity of the pools can be assessed using a 5-day static or shaking batch culture assay. With this assay, limiting dilution cloning can proceed directly from stable pool cell trains.

**Note:** Batch culture (defined as no feed or supplementation during culture) beyond 5 days in CD FortiCHO™ Medium is not recommended.

## Next steps

- To perform clone isolation by limiting dilution to obtain single clones expressing high levels of your protein(s), see page 37.
- To scale up your clones for protein expression, see page 43.

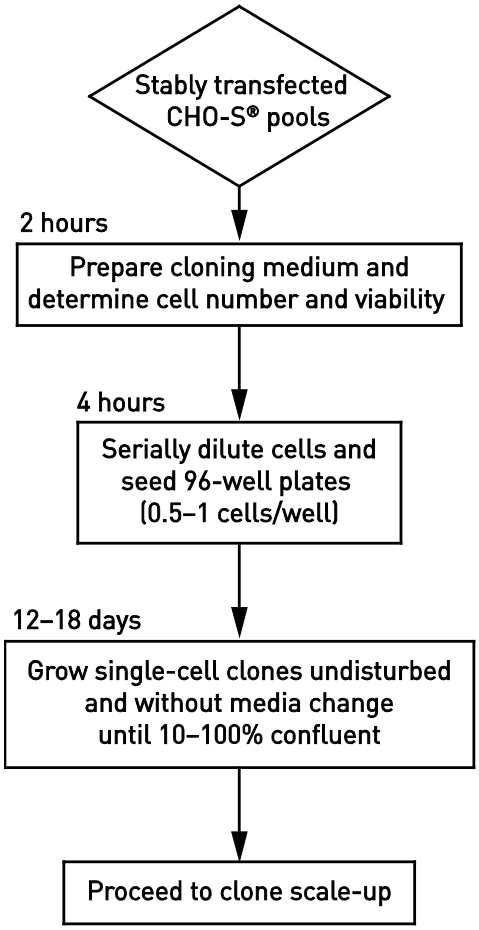
# Isolate clones by limiting dilution

**Introduction**

Development of a CHO cell line for commercial production of a recombinant protein requires clonality of the final cell population. This is achieved by limiting dilution cloning (LDC), or any method to isolate single cells. We provide protocols for LDC since it requires no special equipment.

**Limiting dilution cloning workflow**

The flowchart depicts the major steps to obtain a clonal cell line (i.e., derived from a single cell) by diluting the pool(s) of stably transfected cells to 1 cell or less per well in a 96-well plate containing CD FortiCHO™ Medium. In most cases, a single cell will form a distinct colony that can be scaled up using the procedures described in this section. You may also statistically calculate the desired number of cells per well to help ensure monoclonality, or subclone the top-producing clones.


Stably transfected CHO-S® pools
2 hours
Prepare cloning medium and determine cell number and viability
4 hours
Serially dilute cells and seed 96-well plates (0.5–1 cells/well)
12–18 days
Grow single-cell clones undisturbed and without media change until 10–100% confluent
Proceed to clone scale-up

## Cloning considerations

- When choosing a workflow, consider the amount of protein you wish to produce, your available resources, and the amount of time it will take to obtain your clonal, high-producing cell lines.
- Because the growth rate and protein production of each clone varies, you may need to optimize clone isolation conditions by adjusting the timing of clone scale-up.
- Based on your preferred method of statistical analysis, the number of cells per well can be adjusted to increase the likelihood of producing monoclonal wells. If you choose to seed below 0.5 cells/well, we recommend that you increase the number of 96-well plates you seed to generate your desired number of monoclones. Alternatively, proceed with cloning at 1 cell/well with the intent of subsequently subcloning top-producing clones.
- To achieve the highest cloning efficiency in LDC with CD FortiCHO™ Medium, supplement only with 6 mM L-glutamine. Supplementation with conditioned media or Anti-Clumping Agent is not necessary for LDC with CHO-S™ cells.

  **Note:** Do not use media containing 8 mM L-glutamine for LDC.
- Depending on how many clones you wish to screen before scale-up, you may increase the number of LDC plates according to your anticipated cloning efficiency. The number of clones obtained from a 96-well plate varies depending on the experiment.
- For greatest clone diversity, we recommend seeding multiple stable pools for LDC. For example, if 30–40 plates are desired, seed 10–20 plates per pool if cloning from 2–3 pools and screen an equivalent number of clones per pool. Because the two-phase selection scheme described above will generate up to 4 stable pools per transfection, there should be multiple pools from which to choose for LDC.
- Due to the amount of dilution required to perform LDC and the inherent differences between stable pools, cloning efficiency varies from experiment to experiment and from pool to pool. One advantage of cloning from multiple pools is that it averages out the overall pool-to-pool cloning efficiency.

**IMPORTANT!** When using stable pool cell trains for LDC, be sure to include selective pressure during pool passage.

**Note:** In our experience, we achieve 20–45% cloning efficiency using the conditions as described in the next section.

### Prepare cloning medium

The procedure described below using one 50-mL conical tube is sufficient to seed approximately 200 wells. For greater numbers of wells or plates, increase the number of prepared 50-mL conical tubes accordingly.

**Note:** Because of the dilutions that occur during limiting dilution cloning, there is no need to eliminate Anti-Clumping Agent in the passages prior to cloning.

1. Thaw L-glutamine to be used in preparation of CD FortiCHO™ Medium-based cloning medium.

   **Note:** Cloning medium is CD FortiCHO™ Medium supplemented with 6 mM L-glutamine.

2. For each 100 mL of cloning medium required, aseptically mix the following:

   97 mL of basal CD FortiCHO™ Medium

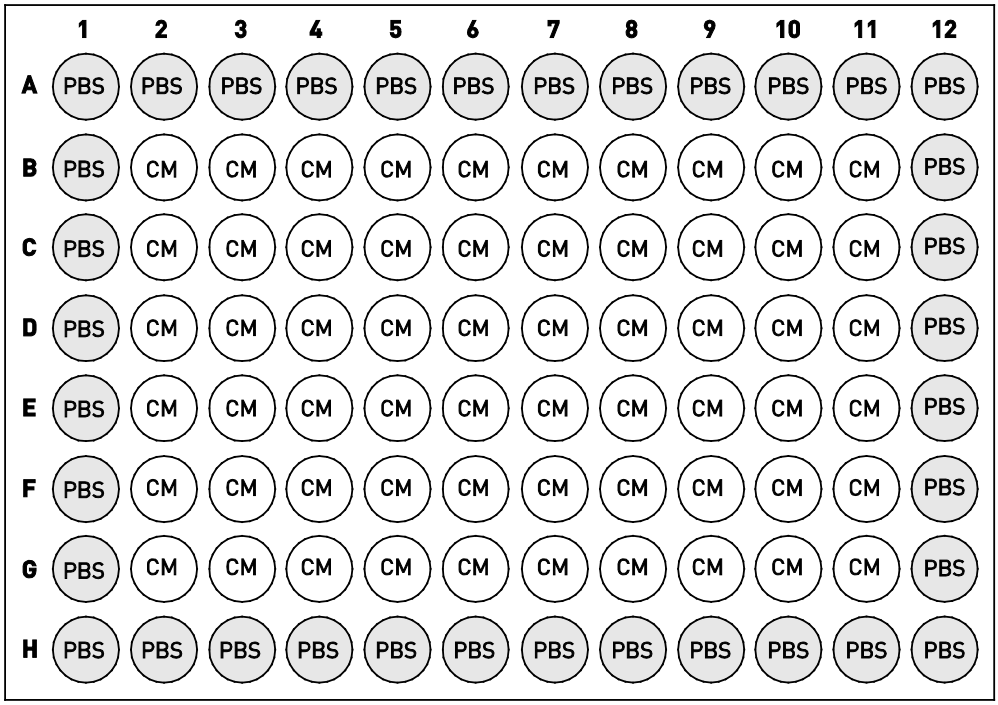
   3 mL of freshly thawed 200 mM L-glutamine

3. For each every 2 × 96-well plate required, add 40 mL of cloning medium to a 50-mL centrifuge tube.
4. Pre-warm the medium at 37°C.

### Setup plate

1. Label a sufficient number of 96-well plates for your procedure (we recommend Falcon 96-well plates, Cat. no. 353072).
2. Optional: Add 200 µL/well of sterile PBS to all peripheral wells to avoid evaporation during incubation (as shown in the figure below).

   Alternatively, peripheral wells can be used for LDC and evaporation can be minimized by setting the incubator humidity to 95%.


1 2 3 4 5 6 7 8 9 10 11 12
A PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS
B PBS CM CM CM CM CM CM CM CM CM CM PBS
C PBS CM CM CM CM CM CM CM CM CM CM PBS
D PBS CM CM CM CM CM CM CM CM CM CM PBS
E PBS CM CM CM CM CM CM CM CM CM CM PBS
F PBS CM CM CM CM CM CM CM CM CM CM PBS
G PBS CM CM CM CM CM CM CM CM CM CM PBS
H PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS PBS

CM = cells diluted in **C**loning **M**edium

## Cell counting and dilution

1. **If using stable pool cell train(s) for limiting dilution cloning**, ensure that the cells are >90% viable before seeding and proceed to step 2.

   **If using frozen stable pool(s) for limiting dilution cloning**, thaw the cells 2–5 days in advance into selection medium lacking Puromycin and MTX; no more than one pass should be needed to reach >90% viability before seeding limiting dilution cloning. We do not recommend extended passage of stable pools in the absence of selection pressure.

2. For each pool, label five 50-mL conical tubes "1" through "5".

3. Use a cell strainer such as BD Biosciences' 40-µm nylon mesh cell strainer, (Cat. no. 352340) to obtain a uniform single-cell suspension with at least 10 mL of your transfected pool CHO-S™ cells into the 50-mL tube labeled "1".

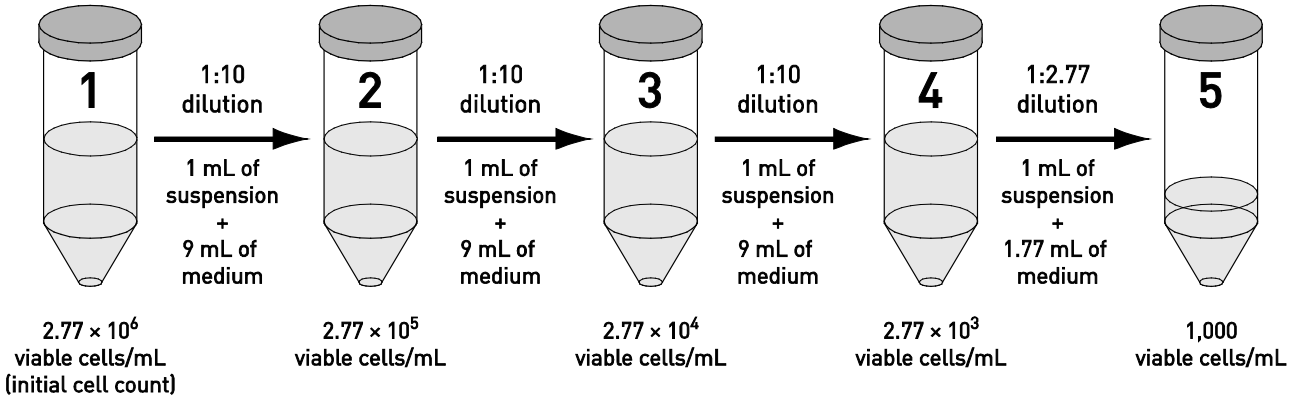
4. Accurately determine the viable cells/mL of the strained pool (see page 19).

5. Serially dilute the cells to a final concentration of ~1,000 viable cells/mL using cloning medium (as shown in figure below).

   **Note:** Mix cells gently after each dilution by gently inverting the capped tube 5 to 6 times. Avoid foaming.

6. Manually count the last dilution (tube 5), and adjusting the volume accordingly in step 2 of **Plate cells**, page 41).

   For example, pipette 8 µL of the cell suspension into 12 wells of a well plate (96 µL total volume). Allow the cells to settle before examining them under the microscope; no staining is required. Counting is facilitated by maintaining the 8 µL as a droplet in each well. If the cells in tube 5 are at 1,000 cells/mL, then you will count 96 cells.


1
1:10 dilution
1 mL of suspension + 9 mL of medium
2
1:10 dilution
1 mL of suspension + 9 mL of medium
3
1:10 dilution
1 mL of suspension + 9 mL of medium
4
1:2.77 dilution
1 mL of suspension + 1.77 mL of medium
5
2.77 × 10^6 viable cells/mL (initial cell count)
2.77 × 10^5 viable cells/mL
2.77 × 10^4 viable cells/mL
2.77 × 10^3 viable cells/mL
1,000 viable cells/mL

Example of serial dilution scheme of cells with initial count of $2.77 \times 10^6$ viable cells/mL

## Plate cells

1. Place the warmed cloning medium (page 39) in the biosafety cabinet.
2. Pipette the necessary volume from "Tube 5" (1,000 cells/mL) into each tube containing 40 mL of cloning medium.

   This brings the final cell density to 5 cells per mL, allowing a seeding density of 1 cell per well when 200 µL of diluted cells is added into each well (see page 40).

   **Note:** If a different seeding density is desired or if manual counting determines that the cells are not at 1,000 cells/mL (step 6 on page 40), adjust the volume transferred from tube 5.

   For example, if manual counting gave 72 cells in 96 µL of total volume, then cells in tube 5 are at 72/96 × 1000 = 750 cells/mL. As such, you would need to add the following volume from tube 5 to 40 mL of cloning medium:

   0.267 mL to achieve 5 cells/mL (for a seeding density of 1 cell/well), OR
   0.134 mL to achieve 2.5 cells/mL (for a seeding density of 0.5 cells/well).

   Always keep the well-seeding volume at 200 µL in step 4 below.
3. Mix the cell suspension gently by inverting the tube and transfer it into a sterile reagent reservoir.
4. Using a multi-channel pipettor, aseptically dispense 200 µL of the diluted cells into each of the empty wells of each 96-well plate (marked as "CM" on the sample plate on page 39).
5. While stacking no more than 5 plates together, incubate the plates undisturbed (without moving or opening the incubator) for at least 12 days at 37°C and 5–8% $CO_2$ in humidified air in a static (non-shaking) incubator.
6. No earlier than day 12 of incubation, examine the wells visually using a microscope for growth of monoclonal colonies.
7. *Optional*: On day 13 or 14, feed all wells with 25–50 µL of complete CD FortiCHO™ Medium (with or without the Anti-Clumping Agent at 1:100 dilution).

   **Note:** The purpose of this feed is to ensure that there is sufficient volume in the wells for both the primary screen assay and the clone scale-up procedures, and it depends on the amount of evaporation your plates have experienced within your static incubator. Feeding may be unnecessary if evaporation is minimized (for example, by setting incubator humidity to 95%).
8. Perform your primary screen (i.e., protein assay of choice) on day 17 or 18 to identify clones of interest.
9. Calculate the percent cloning efficiency.

### Calculate cloning efficiency

Calculate the percent cloning efficiency, normalized at 1 cell per well, as follows:

$$\text{Cloning Efficiency} = \frac{\text{Number of wells showing growth}}{\text{Number of wells seeded} \times \text{cells per well seeded}} \times 100\%$$

For example, the cloning efficiency with 120 colonies growing out of total 600 seeded wells (10 plates with 60 seeded wells/plate) at a seeding density of 0.5 cell per well is 40%.

**Note:** We have achieved 20–45% cloning efficiency under these conditions. The use of the cell strainer prior to diluting cells greatly improves the frequency of monoclonality; however, the observed frequency of monoclonality will vary from experiment to experiment, and will decrease as seeding density increases.

### Next steps

After testing these clones for high levels of protein production using your method of choice, you can proceed to **Clone scale-up and screening**, page 43.

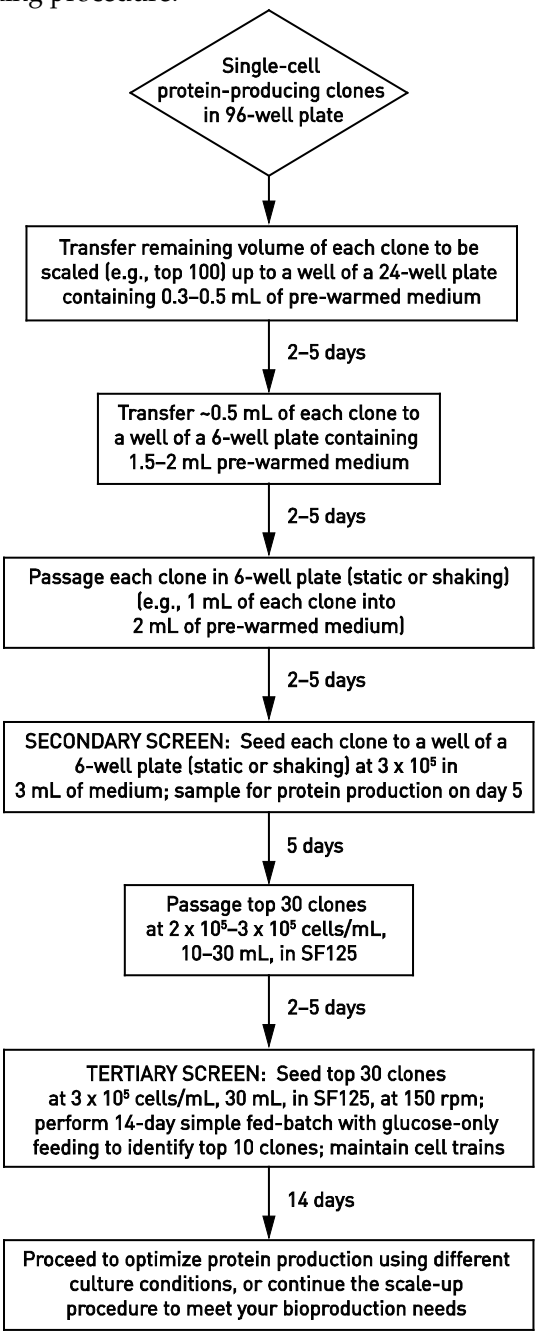
# Clone scale-up and screening

## Introduction

After isolating your clones of interest (previous section), transfer single-cell colonies from 96-well plates to 24 well plates in clone growth medium. Scale up the volume of cells every 2–5 days by transferring each clone into the next larger plate or vessel (i.e., 24-well plates to 6 well plates or T-25 flasks, and then to 125-mL shaker flasks). We recommend performing a secondary screen in 6-well plates, and a tertiary screen in shaker flasks.

## Clone scale-up and screening workflow

The flowchart below depicts a suggested workflow for the major steps in clone scale-up and screening procedure.


Single-cell protein-producing clones in 96-well plate
Transfer remaining volume of each clone to be scaled (e.g., top 100) up to a well of a 24-well plate containing 0.3–0.5 mL of pre-warmed medium
2–5 days
Transfer ~0.5 mL of each clone to a well of a 6-well plate containing 1.5–2 mL pre-warmed medium
2–5 days
Passage each clone in 6-well plate (static or shaking) (e.g., 1 mL of each clone into 2 mL of pre-warmed medium)
2–5 days
SECONDARY SCREEN: Seed each clone to a well of a 6-well plate (static or shaking) at 3 x 10^5 in 3 mL of medium; sample for protein production on day 5
5 days
Passage top 30 clones at 2 x 10^5–3 x 10^5 cells/mL, 10–30 mL, in SF125
2–5 days
TERTIARY SCREEN: Seed top 30 clones at 3 x 10^5 cells/mL, 30 mL, in SF125, at 150 rpm; perform 14-day simple fed-batch with glucose-only feeding to identify top 10 clones; maintain cell trains
14 days
Proceed to optimize protein production using different culture conditions, or continue the scale-up procedure to meet your bioproduction needs

### Guidelines for clone scale-up

- The total clone scale-up process from a 96-well plate to a 125-mL flask takes 2–3 weeks, depending on the growth rate of each clone. Assess the protein production of each clone using your method of choice (see below or refer to **Protein production**, page 36) and carry forward the top-producing clones to the next stage in the scale-up process.
- We have observed that clones that are ≥10% confluent on day 17 or 18 post seeding in 96-well plates will readily scale up. However, clones <10% confluent should also be scaled up because they can potentially score as high protein producers. If desired, clones <10% confluent can be allowed to grow for an additional 3–5 days and re-screened for protein production.
- We have had success scaling clones from 96-well plates to 24-well plates to 6-well plates, followed by an assessment of clone productivity after 2 passages in 6-well plates. This is sufficient for the vast majority of clones to be >90% viable before the secondary screen.
- For the secondary screen, we suggest seeding all clones at $3 \times 10^5$ viable cells/mL in a 3-mL volume in 6-well plates and assessing protein productivity at day 5 post-seeding. After assessing productivity, we recommend counting and scaling up the top 30 clones from 6 well plates to shake flasks. At this stage, do not let the clones grow more than 4 days without passaging to prevent their health from becoming compromised.
- For best results in secondary screen, we recommend that the second 6-well plate passage as well as the secondary screen itself be performed using shaking 6-well plates. This requires the plates to be incubated in a box, which can be done inexpensively by purchasing a plastic storage box compatible with your 6-well plate configuration and incubator space, and puncturing the lid with multiple holes to allow for gas exchange. We generated our data using 13 × 10 × 5.5 inches plastic boxes shaking at 130 rpm. As with static plates, shaking plates should not be stacked more than five high. If using shaking 6-well plates is not desired, we have found that both static and shaking 6-well plates can be used to eliminate low producers during the secondary screen.

### Required materials

- Single cell-derived protein-producing clones in 96-well plates
- Clone Growth Medium (complete CD FortiCHO™ Medium and Anti-Clumping Agent at a 1:100 dilution)
- Sterile tissue culture dishes (24-well and 6-well) , and sterile 125-mL polycarbonate shaker flasks
- Non-shaking static incubator set at 37°C, ≥80% humidified atmosphere, and 8% $CO_2$
- Shaking incubator set at 37°C, 70–80% humidified atmosphere, and 8% $CO_2$, shaking at 130–150 rpm
- Assay for determining protein production

## Protocol

1. When individual clones are 10–90% confluent in 96-well plates (day 17 or 18 post-seeding), aseptically harvest the desired clones by pipetting the cells up and down gently and transferring the entire content of each well into a separate well of a 24-well plate containing 0.3–0.5 mL of fresh Clone Growth Medium (complete CD FortiCHO™ Medium and Anti-Clumping Agent at a 1:100 dilution).

   **Note:** Until clones are safely frozen down, we recommend adding fresh medium to the wells from which cells were scaled up at each stage as back-up.

2. After 2–5 days, transfer the desired clones into 6-well plates using the same procedure. The final culture volume in a 6-well plate is 2–3 mL.

3. Perform a second passage, such as a 1:5 dilution, into new 6-well plates to ensure that the vast majority of the clones are >90% viable before performing the secondary screen. Ideally this passage in 6-well plate is when shaking conditions are reintroduced, but the plates can also be incubated without shaking; see above Guidelines for details.

   **Note:** We recommend performing the second passage in a shaking 6-well plate at 130 rpm as described on the preceding page; however, static 6-well plates also work. Ideally this passage in 6-well plate is when shaking conditions are reintroduced, but the plates can also be incubated without shaking; see above Guidelines for details.

4. Set up the secondary screen in static or shaking 6-well plates (consistent with the conditions used in the preceding step). Seed the cells at $3 \times 10^5$ viable cells/mL in 3 mL of Clone Growth Medium **supplemented with 3 g/L glucose** and incubate for 5 days before sampling for productivity.

   **Note:** As with the preceding step, you may use either static or shaking 6-well plates, but we have seen better signal-to-noise ratio and cell growth when using shaking 6-well plates.

   **Note:** Fast-growing clones may starve during the 5-day incubation period, which is why we recommend supplementing the medium with additional glucose. For example, if you are working with a 45% sterile glucose solution and need 300 mL of fresh medium, add 2 mL glucose to 300 mL of Clone Growth Medium before using to seed cells in 6-well plates.

5. On day 5 post-seeding:

   a. Determine the titer within each well using your protein assay of choice.
   b. Count and passage the desired number of top-producing clones into 125-mL shaker flasks in 15-30 mL of Clone Growth Medium.

6. Once the clones are expanded to 125-mL shaker flasks, incubate the cells at 37ºC and 8% $CO_2$, with shaking at 130–150 rpm and passage every 2–3 days. **For best results, use the rpm speed that was used during stable pool selection**.

7. As soon as there are sufficient cells, freeze down a mini-RCB of each top-producing clone.

8. Perform a tertiary screen with the top-producing clones in 125-mL shaker flasks. We recommend using a 14-day simple fed-batch in Clone Growth Medium:
   i. Sample cultures daily or at regular intervals (e.g., on days 0, 3, 5, 7, 10, 12, and 14) to determine the cell density, viability, and productivity until culture viability drops below 50% or day 14 of culture is reached.
   ii. Feed with glucose, after sampling, as follows:
      - Day 3: add 4 g/L of glucose
      - Day 5: add 4 g/L of glucose
      - Day 7: add 6 g/L glucose

   **Note:** We recommend passaging clones at least two times in shaker flasks before initiating the tertiary screen to ensure that the clones' doubling times have stabilized.

**IMPORTANT!** Prepare frozen cell stocks (at least 5 vials per clone) prior to optimizing protein production. You may then optimize protein production using different culture conditions, such as including EfficientFeed™ C + AGT™ Supplement, or continue the scale-up procedure to meet your bioproduction needs.

# Stability and fed-batch assessments of top clones

## Guidelines for research cell bank and stability assessment in Dynamis™ Medium

After identifying your top-producing clones, we recommend transitioning your clones for all subsequent steps (from RCB generation all the way through to final protein production) into Dynamis™ Medium, our next-generation production medium. Dynamis™ Medium is available in both liquid and AGT™ formats, and is the medium of choice for large-scale protein production with Freedom™ CHO-S™ clones. Clones from the Freedom™ CHO-S™ workflow can be thawed directly and seamlessly into Dynamis™ Medium.

**Note:** The starting formulation for DynamisTM Medium is the same as CD FortiCHO™ Medium, 8 mM L-glutamine and 1:100 Anti-Clumping Agent, but the levels of both of these supplements can be adjusted as needed to optimize for your desired protein production and quality.

1. Create a large cell bank of each clone for which productivity and stability will be assessed in Dynamis™ Medium.

   **Note:** Clones frozen down during clone screening can be directly thawed into Dynamis™ Medium supplemented with 8 mM L-glutamine and 1:100 Anti-Clumping Agent without any negative effects.

   **Note:** We recommend that you use the Dynamis™ Medium cell bank for stability assessment, preliminary cell sterility testing, fed-batch and process optimization, and subsequent cell banking needs.

   **Note:** We recommend that at least 10 clones be assessed for production stability.

2. Determine stability of desired number of clones over the projected time needed to scale up from RCB to your final protein production vessel.

   For example, we typically perform stability assessment over 60 generations, which is more than enough time to allow for clone scale-up from an RCB to a 10,000 L production tank. Adjust as needed to meet your scale-up process needs.

3. Optimize protein production and product quality using Dynamis™ Medium, EfficientFeed™ C+ AGT™ Supplement, and process development. While we provide a sample feeding schedule below, each clone could have unique feed needs, and thus the feeding schedule provided is just a relative starting point.

   **Note:** For additional support, or customized medium and feed development to optimize protein production and/or product quality, please contact Gibco™ Bioprocess Services.

### Sample stability assessment protocol

With a 60-generation stopping point, stability assessment takes ~6 weeks for clones with doubling times of ~17 hours. Use Dynamis™ Medium supplemented with 8 mM L-glutamine and 1:100 Anti-Clumping Agent as the medium for all subsequent steps.

1. Thaw each clone into a 125-mL shaker flask, and incubate as previous (37°C, 8% $CO_2$, and 80% humidity while shaking at 150 rpm).
2. Passage on a regular schedule, such as every 3 days or on a 3/4-day schedule, seeding at $1 \times 10^5$–$2 \times 10^5$ viable cells/mL.
3. *Optional*: At first passage, add 100 nM MTX to each flask for this and all subsequent passages.

   Since some customers find it acceptable to include MTX during clone scale-up prior to production, we have provided the recommended conditions here. We have established that most Freedom™ CHO-S™ clones will be stable (defined as maintaining titer at ≥70% initial values) over 60 generations with a minimal amount of MTX (100 nM), regardless of the final selection pressure that was used to generate the pool from which the clone was isolated.
4. Tracking clone productivity: After seeding the new passage flask, feed the cells remaining in the old passage flask with 5 g/L glucose and incubate the flask to day 7 before sampling for protein titer/quality.

   While we have used this procedure to assess productivity once a week, the productivity trend will be more accurate with more data points, so the glucose feeding and sampling schedule can be used with every passage flask.
5. Continue passage and day 7 productivity assessments as long as is needed to simulate the time of your scale-up process.
6. *Optional*: run productivity assessments as full 14-day simple fed-batch (see page 36).
7. *Best practice*: Every 20–30 generations, freeze at least 5 vials of each clone as back-up.

### Sample feeding schedule for EfficientFeed™ C+ AGT™ Supplement and Dynamis™ Medium

We recommend using EfficientFeed™ C+ AGT™ Supplement with Dynamis™ Medium. Compared to the original CD EfficientFeed™ C AGT™ Nutrient Supplement, the EfficientFeed™ C+ AGT™ Supplement contains components that are uniquely combined to allow for simple pH-neutral reconstitution, as well as the ability to super-concentrate the supplement from 81.2 g/L (1X) to 162.4 g/L (2X) for reduction of product dilution and increased titers with additional feeding.

1. Thaw each clone using Dynamis™ Medium and a 125-mL shaker flask, and incubate as previous (37°C, 8% $CO_2$, and 80% humidity while shaking at 150 rpm).
2. Passage at least twice before seeding a 250-mL shaker flask at $3 \times 10^5$ viable cells/mL in 60 mL of Dynamis™ Medium.
3. Sample for cell counts on day 0.
4. Sample daily beginning on day 3 for metabolites, titer/product quality, and cell counts.
5. Feed glucose up to 6 g/L when levels are below 2 g/L.

6. Feed CD EfficientFeed™ C+ AGT™ Supplement Plus as follows:

| EFC+ concentration | Culture day | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1X | | | | 10% | | 10% | | 10% | | | 10% |
| 2X | | | | 5% | | 5% | | 5% | | | 5% |

Using the above protocol, viability can be readily maintained above 50% through day 17 or longer if your protein of interest's quantity or quality does not degrade with extended incubation. Additional feeding schedules may also need to be tested to find the optimal feed process for your lead clone(s), but the above protocol provides a starting point for measuring feed responsiveness. Clone feed screening will also generate material for Spent Media Analysis analytical service and other media optimization and process development services available through Gibco™ Bioprocess Services.

# Appendix A

## Troubleshooting

In addition to a few common problems highlighted below, the most commonly asked questions regarding the Freedom™ CHO-S™ Kit have been compiled for reference into the Freedom™ CHO-S™ FAQ eBook: **http://www.life-technologies.uberflip.com/i/330570**

### Thawing CHO-S™ Cells (cGMP-banked)

The table below lists some potential problems and possible solutions that may help you troubleshoot your cell culture experiment.

| Observation | Possible cause | Recommended action |
|---|---|---|
| No viable cells after thawing original vial | Cells not stored correctly | Order new cell stock and store in liquid nitrogen vapor phase. Keep in liquid nitrogen vapor phase until thawing. |
| | Incorrect thawing medium or method | • Use pre-warmed CD FortiCHO™ Medium supplemented with 8 mM L-glutamine.<br>• Do not add antibiotics to medium because it may negatively impact cell growth.<br>• Incubate cultures on an orbital shaker set at 130–150 rpm in a 37°C incubator with a 70–80% humidified atmosphere and 8% $CO_2$. |
| No viable cells after thawing stocks | Cells not frozen correctly | Follow the protocol on page 20 to freeze cells. |
| | Incorrect thawing medium or method | • Use pre-warmed CD FortiCHO™ Medium supplemented with 8 mM L-glutamine.<br>• Do not add antibiotics to medium because it may negatively impact cell growth.<br>• Incubate cultures on an orbital shaker set at 130–150 rpm in a 37°C incubator with a 70–80% humidified atmosphere and 8% $CO_2$. |

**CulturingCHO-S™ cells (cGMP-banked**

The table below lists some potential problems and possible solutions that may help you troubleshoot your cell culture experiment.

| Observation | Possible cause | Recommended action |
|---|---|---|
| Cells grow slowly | Incorrect growth medium | Use pre-warmed CD FortiCHO™ Medium supplemented with 8 mM L-glutamine. |
| | Shaker not set up properly | Shake on an orbital shaker at 130–150 rpm in 37°C incubator with a humidified atmosphere of 8% $CO_2$. |
| | Medium is foamy | • Keep shaker speed at 130–150 rpm.<br>• At larger culture scales, you may add FoamAway™ Irradiated AOF to prevent foaming, see page 64. |
| | Cells too old | Use healthy CHO-S™ Cells (cGMP-banked) under generation 25; do not overgrow. |
| | Cell culture clumpy | • Provide agitation to the culture, a regular and frequent cell passage schedule, and maintenance of cells at recommended densities (<$2 \times 10^6$ cells/mL).<br>• Use Anti-Clumping Agent, see page 64. **Do not** use Anti-Clumping Agent during transfection; Anti-Clumping Agent should to be removed 2 passages before transfection.<br>• Use a cell strainer (BD Biosciences, 40 µM nylon mesh, Cat. no. 352340) before transfection and before seeding for LDC |
| | Cells overheat | Calibrate the incubator by comparing the actual temperature of the medium in a culture flask while shaking on the platform to the temperature setting of the incubator. Use a flask containing growth medium, but no cells. Then adjust the incubator setting to the defined temperature. |

## Transfection

The table below lists some potential problems and possible solutions that may help you troubleshoot your transfection experiments.

| Observation | Possible cause | Recommended action |
|---|---|---|
| Very few or no stably-transfected cells obtained | Improperly cultured cells | • Exactly follow procedures as outlined in **Thaw and subculture CHO-S™ Cells (cGMP-banked)**, page 18.<br>• Thaw a new vial of early-passage cells.<br>• Do not add antibiotics during transfection.<br>• Always include Anti-Clumping Agent in your selection medium |
| | Cells not passed 24 hours before transfection | Approximately 24 hours before transfection, pass cells at $5 \times 10^5$ cells/mL. |
| | FreeStyle™ Max Reagent handled incorrectly | • Store at 4°C. **Do not freeze.**<br>• Mix gently by inversion. **Do not vortex.** |
| | Used poor quality expression construct plasmid DNA (i.e., plasmid DNA from a mini-prep) | **Do not** use miniprep plasmid DNA for transfection. Prepare midiprep plasmid DNA with low or no endotoxin contamination. |
| | DNA contaminated | Sterilize DNA using a 0.22-µm filter. |
| | DNA precipitated upon addition of FreeStyle™ MAX | • To ensure that it is free of salt and organic solvents, do **not** purify the DNA following its linearization with *Nru* I, before mixing the DNA with FreeStyle™ MAX for transfection.<br>• Be sure to add FreeStyle™ MAX to DNA, and that the addition is made with the tip immersed in the DNA solution with mixing, not drop-wise to the top. |
| | Cells did not recover from selection in CD FortiCHO™ Medium containing Puromycin and MTX | The amounts of Puromycin and MTX required for selection of resistant cells vary with a number of factors, including the identity of the protein(s) being expressed. Try reducing the amount of Puromycin and MTX used in Selection Phase 1. |

## Protein expression

The table below lists some potential problems and possible solutions that may help you troubleshoot your protein expression levels.

| Observation | Possible cause | Recommended action |
|---|---|---|
| No or low protein detected in the supernatant after transient or stable transfection | Gene of interest does not contain Kozak translation initiation sequence | Add a Kozak consensus site to the forward PCR primer, resynthesize your gene(s) of interest. |
| | Premature stop codons | Remove stop codons by your method of choice. |
| | Improper or ineffective secretion signal | Replace secretion signal. Use endogenous secretion signal if possible. |
| | Genes not optimized | Have your genes optimized, not only for CHO codons, but also for cryptic splice sites, RNA secondary structure, killer motifs, etc. We highly recommend GeneArt™ GeneOptimizer™ Process for Multi-parameter Gene Optimization |

## Clone selection

The table below lists some potential problems and possible solutions that may help you troubleshoot your clone selection experiments.

| Observation | Possible cause | Recommended action |
|---|---|---|
| Very few or no single cell clones obtained after cloning | Error in cell counting and dilution during limiting dilution cloning | • Follow procedures exactly as outlined in **Cell counting and dilution**, page 40.<br>• Dilute cells no more than 1:10 with a very small amount (<1mL) of cell suspension. |
| | Plates moved too soon after seeding the cells | Seeded plates should be incubated undisturbed for at least 12 days. |
| | Improper medium used | **Prepare Cloning Medium** as described on page 39, CD FortiCHO™ Medium supplemented with 6 mM L-glutamine. No additional supplements are required. |

# Appendix B

## Quick reference protocols

### Introduction

The following pages contain one-page quick reference protocols for transfecting CHO-S™ Cells for stable protein expression using either the FreeStyle™ MAX Reagent (page 55) or the Neon™ Transfection System (page 56) and detailed workflow diagrams for Phase I and Phase II of the two-phase selection scheme to generate a pool of stably-transfected cells (pages 57–58). Each quick reference protocol consists of an outline of the necessary steps and space for notes that you can then transfer to your laboratory notebook.

The quick reference protocols and the workflow diagrams are intended for experienced users only. These protocols and workflow diagrams provide summary instructions for transfecting CHO-S™ Cells and selecting stably-transfected cells; they do **not** provide detailed instructions to successfully perform the steps necessary for expressing your protein of interest.

Detailed protocols for transfecting CHO-S™ Cells using the FreeStyle™ MAX Reagent are found on page 23 and using the Neon™ Transfection System on page 27. Detailed instructions for the two-phase selection scheme are found on pages 31–35. We recommend that you familiarize yourself with the detailed protocols before starting your experiments.

# FreeStyle™ MAX transfection protocol and calculations

**Cell culture before transfection:** minimum 5 passages, viability ≥ 95%

**Note:** The numbering in this section does not match the numbering within the detailed protocol.

| | |
|---|---|
| 1. Count cells (after cell straining if needed). | 1. a. Cell count (cells/mL):_____<br>b. Viability (%):_____ |
| 2. Determine volume needed for $3 \times 10^7$ cells (per transfection). Dilute cells with pre-warmed complete medium to $1 \times 10^6$ cells/mL in 30 mL; maintain with shaking at 37°C until needed.<br>Note: Do not centrifuge cells prior to transfection. | 2. a. mL cells ($3 \times 10^7 \div$ 1a):_____<br>b. mL fresh medium (30 – 2a):_____ |
| 3. Dilute 50 µg of plasmid DNA in 1.5 mL final volume of OptiPRO™ SFM. | 3. a. Plasmid ID:_____<br>b. Concentration (µg/µL):_____<br>c. µL DNA for 50 µg (50 ÷ 3b):_____<br>d. µL OptiPRO™ SFM (1500 – 3c):_____ |
| 4. Dilute 50 µL FreeStyle™ MAX in 1.45 mL of OptiPRO™ SFM for each transfection; mix without vortexing. | |
| 5. With tip submerged, slowly add 1.5 mL diluted FreeStyle™ MAX to diluted DNA; incubate 10–20 minutes at room temperature to allow for DNA-FreeStyle™ MAX complex formation.<br>**Note:** If precipitation is observed, start over from step 3. | 5. DNA + FreeStyle MAX complex formation:<br>Incubation start time:_____<br>Incubation end time:_____ |
| 6. Add all 3 mL of FreeStyle™ MAX-DNA mixture dropwise to cells from step 2 while swirling. | |
| 7. Incubate with shaking at 37°C in a humidified incubator with 8% $CO_2$ for 2 days and proceed to **Select stable transfectants for protein expression**, page 31. | |

# Neon™ transfection protocol and calculations

**Cell culture before transfection:** minimum 5 passages, viability ≥ 95%
**Note:** The numbering in this section does not match the numbering within the detailed protocol.

**Prepare Neon™ Transfection System:** Set desired voltage, pulse width, and pulse number (record at step 7); fill the Neon™ Tube with 3 mL Electrolytic Buffer E2 before inserting it into the Neon™ Pipette Station.

1. Count cells (after cell straining if needed). Determine volume needed for harvesting $4 \times 10^7$ cells by centrifugation.
2. Centrifuge cells, discard supernatant, and resuspend with at least 20 mL PBS to wash (Wash 1).
3. Centrifuge cells, discard supernatant, and resuspend with at least 20 mL PBS to wash (Wash 2).
4. Recount cells prior to centrifuging Wash 2.
5. Discard supernatant and resuspend cells to $5 \times 10^7$ cells/mL in Resuspension Buffer R.
6. Mix cells with DNA: generate four tubes containing 120 µL cells + 12 µg DNA

   OR

   generate one tube containing 480 µL cells + 48 µg DNA.
7. Without introducing air, draw up 100 µL of cell-DNA mixture into the Neon™ Tip, electroporate, and immediately proceed to step 8.
8. Dispense cells into a T-75 flask containing 10 mL of pre-warmed complete medium (for a total of two T-flasks).
9. Repeat steps 7 and 8 three more times per plasmid, with two repeats dispensed per T-flask, for a total of four repeats and two T-flasks.
10. Incubate T-flasks at 37°C in a humidified incubator with 8% $CO_2$ for 2 days, pool duplicate T-75 flasks, and proceed to **Select stable transfectants for protein expression**, page 31.

1. a. Cell count (cells/mL):_____

   b. ml cells to centrifuge
   ($4 \times 10^7 \div$ 1a) _____
2. Wash 1 (mL):_____
3. Wash 2 (mL):_____
4. a. Recount (cells/ mL):_____

   b. Total cells (4a × mL Wash 2):_____
5. mL Resuspension Buffer R
   (4b ÷ $5 \times 10^7$):_____
6. Plasmid ID:_____

   Concentration (µg/µL):_____

   µL cells (120 or 480 µL):_____

   µL DNA (12 or 48 µg ÷ conc.):_____
7. Neon™ electroporation settings:

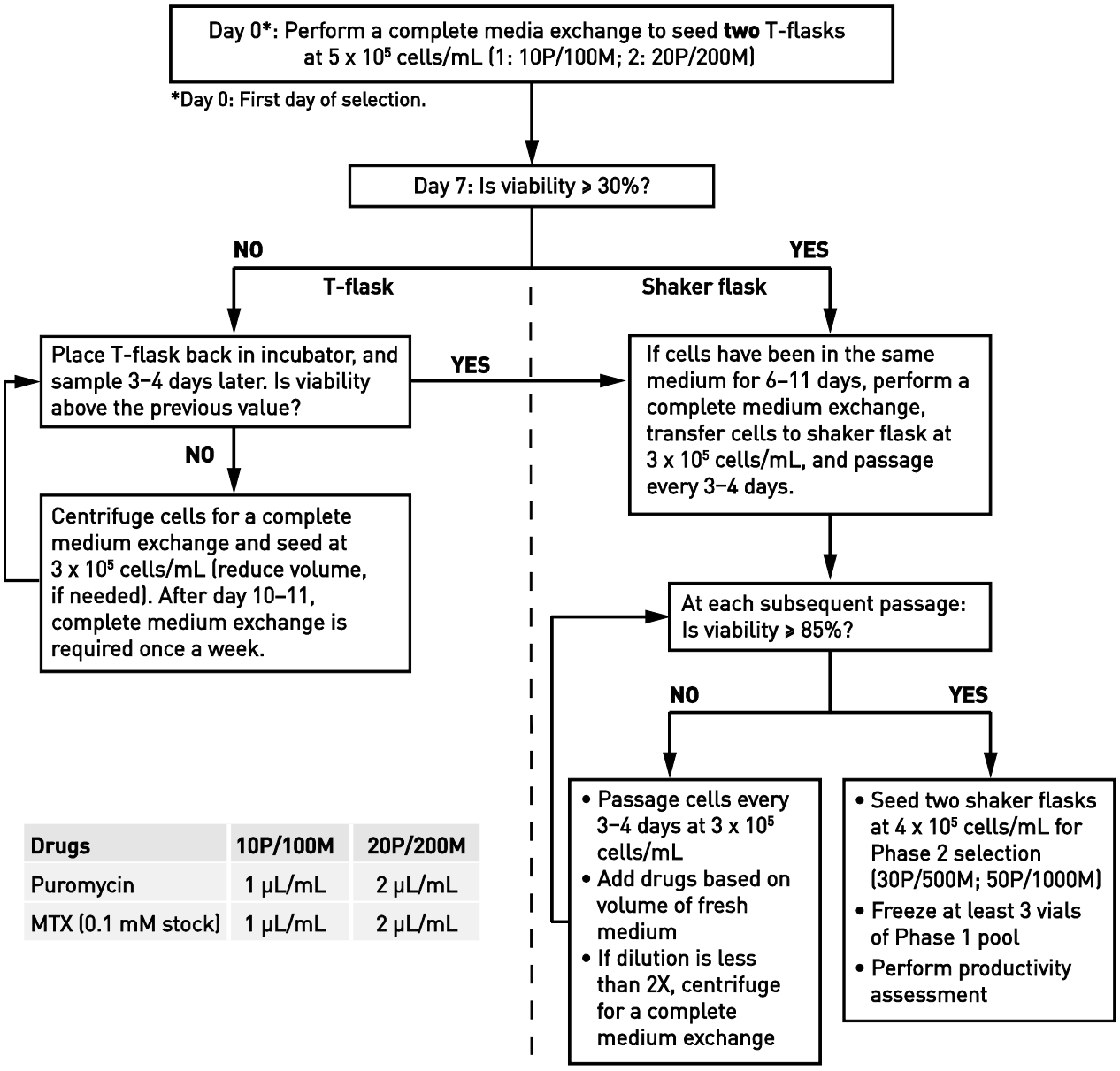
   Pulse voltage (V):_____

   Pulse width (ms):_____

   Number of pulses:_____

# Phase 1 selection

**Each time cells are handled, strain cells before counting if clumping is observed.**

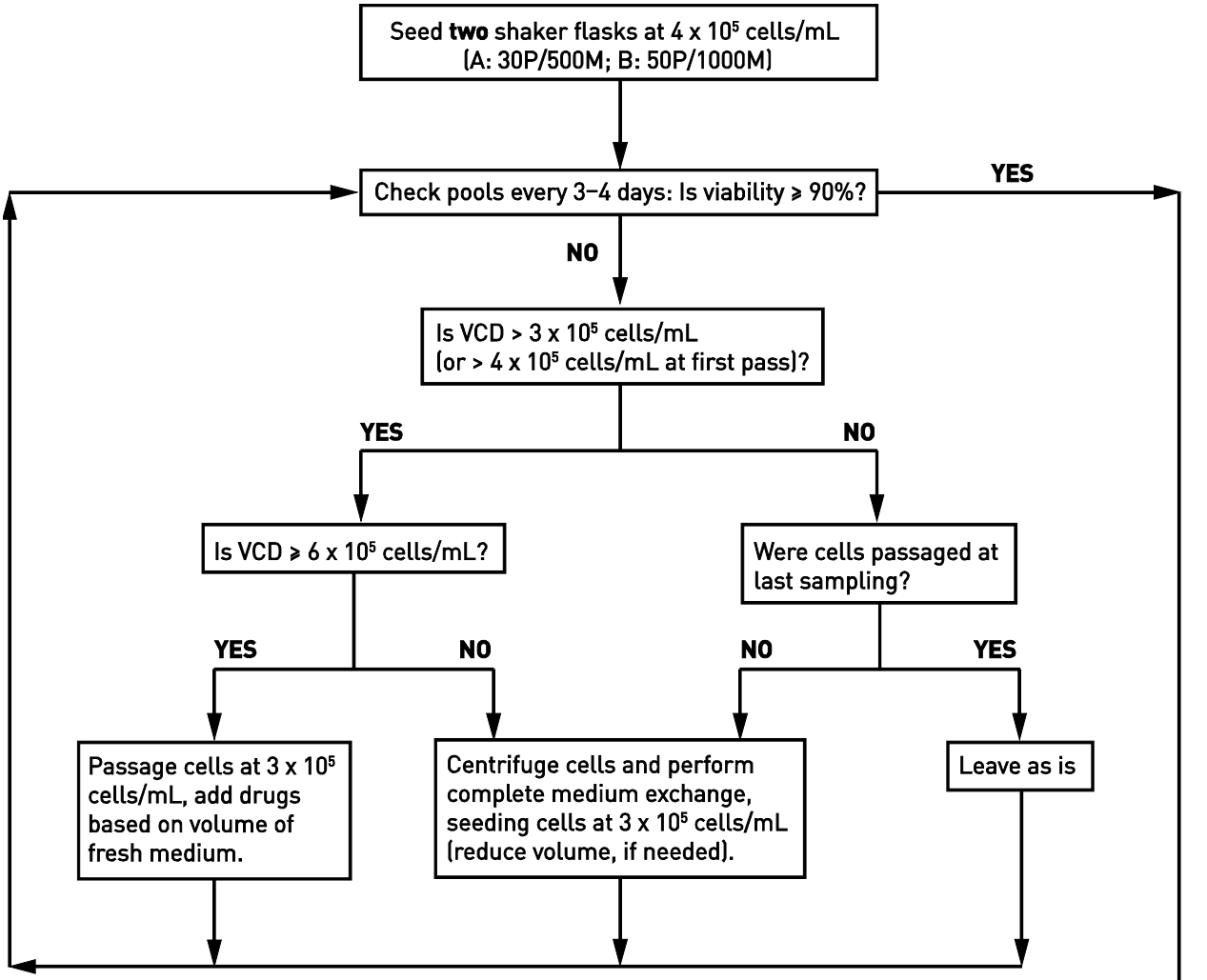

Day 0*: Perform a complete media exchange to seed two T-flasks at 5 x 10^5 cells/mL (1: 10P/100M; 2: 20P/200M)
*Day 0: First day of selection.
Day 7: Is viability ≥ 30%?
NO
YES
T-flask
Shaker flask
Place T-flask back in incubator, and sample 3–4 days later. Is viability above the previous value?
YES
NO
Centrifuge cells for a complete medium exchange and seed at 3 x 10^5 cells/mL (reduce volume, if needed). After day 10–11, complete medium exchange is required once a week.
If cells have been in the same medium for 6–11 days, perform a complete medium exchange, transfer cells to shaker flask at 3 x 10^5 cells/mL, and passage every 3–4 days.
At each subsequent passage: Is viability ≥ 85%?
NO
YES
• Passage cells every 3–4 days at 3 x 10^5 cells/mL
• Add drugs based on volume of fresh medium
• If dilution is less than 2X, centrifuge for a complete medium exchange
• Seed two shaker flasks at 4 x 10^5 cells/mL for Phase 2 selection (30P/500M; 50P/1000M)
• Freeze at least 3 vials of Phase 1 pool
• Perform productivity assessment

| Drugs | 10P/100M | 20P/200M |
|---|---|---|
| Puromycin | 1 µL/mL | 2 µL/mL |
| MTX (0.1 mM stock) | 1 µL/mL | 2 µL/mL |

## Phase 2 selection

**Each time cells are handled, strain cells before counting if clumping is observed.**

**Check pools every 3 – 4 days.**


Seed two shaker flasks at 4 x 10^5 cells/mL (A: 30P/500M; B: 50P/1000M)
Check pools every 3–4 days: Is viability ≥ 90%?
YES
NO
Is VCD > 3 x 10^5 cells/mL (or > 4 x 10^5 cells/mL at first pass)?
YES
NO
Is VCD ≥ 6 x 10^5 cells/mL?
Were cells passaged at last sampling?
YES
NO
NO
YES
Passage cells at 3 x 10^5 cells/mL, add drugs based on volume of fresh medium.
Centrifuge cells and perform complete medium exchange, seeding cells at 3 x 10^5 cells/mL (reduce volume, if needed).
Leave as is
• Freeze at least 5 vials.
• Perform productivity assessment.

| Drugs | 30P/500M | 50P/1,000M |
|---|---|---|
| Puromycin | 3 µL/mL | 5 µL/mL |
| MTX (**1 mM stock**) | 0.5 µL/mL | 1 µL/mL |

# Appendix C

## Media formulations

### Introduction

The table below lists the various media formulations required for the development of stable cell lines for protein production using the Freedom™ CHO-S™ Kit. All the media formulations use CD FortiCHO™ Medium as the basal medium.

**Note:** We do **not** recommend using L-glutamine, or medium supplemented with L-glutamine, beyond one month.

| Medium | Basal medium | Final concentration | | Additional supplements |
|---|---|---|---|---|
| | | L-glutamine | Anti-Clumping Agent | |
| Complete medium* | CD FortiCHO™ Medium | 8 mM | — | — |
| Selection medium | | 8 mM | 1:100 dilution | Puromycin + MTX |
| Cloning medium | | 6 mM | — | — |
| Clone growth medium** | | 8 mM | 1:100 dilution | — |
| Clone productivity medium† | | 8 mM | 1:100 –1:100 dilution | — |
| Freezing medium | | 8 mM | optional | 10% DMSO |

* Used for thawing and passaging parental CHO-S™ cells.

** Used for clone scale-up.

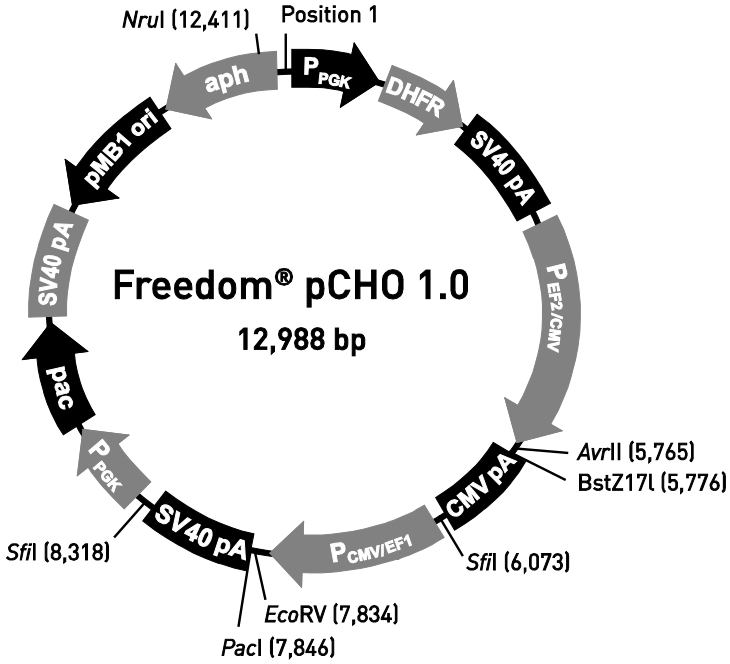
† Used for assessing clone productivity; inclusion of lower concentrations of Anti-Clumping Agent may be desired for protein purification. Inclusion of higher concentrations of Anti-Clumping Agent, however, may improve the productivity of some clones.

# Appendix D

## Map and features of Freedom™ pCHO 1.0 vector

### Map

The map below shows the elements of the Freedom™ pCHO 1.0 vector. See page 61for detailed descriptions of the elements of the Freedom™ pCHO 1.0 vector.




**Comments for Freedom® pCHO 1.0**
**12,988 nucleotides**

| | |
|---|---|
| PGK (phosphoglycerate kinase) promoter: | 117–623 |
| DHFR (dihydrofolate reductase) gene: | 730–1,293 |
| SV40 polyadenylation signal: | 1,404–1,631 |
| EF2/CMV hybrid promoter: | 1,728–5,517 |
| CMV polyadenylation signal: | 5,773–6,052 |
| CMV/EF1 hybrid promoter: | 6,093–7,565 |
| SV40 polyadenylation signal: | 7,848–8,094 |
| PGK (phosphoglycerate kinase) promoter: | 8,351–8,839 |
| Puromycin resistance gene (pac): | 8,860–9,459 |
| SV40 polyadenylation signal: | 9,583–9,823 |
| pMB1 origin of replication: | 10,495–11,168 (c) |
| Kanamycin resistance gene (aph): | 11,684–12,499 (c) |

(c) = complementary strand

## Features

The Freedom™ pCHO 1.0 vector contains the following elements. Features have been functionally tested, and the vector has been fully sequenced.

| Feature | Benefit |
|---|---|
| EF2/cytomegalovirus (CMV) hybrid promoter | Allows efficient, high-level expression of your recombinant protein |
| *Avr*II and *Bst*Z17I restriction enzyme sites | Entry points for gene insertion behind the EF2/CMV promoter (see page 18 for sequencing primers used for analyzing EF2/CMV promoter-gene of interest junction) |
| CMV polyadenylation signal | Allows efficient transcription termination and polyadenylation of mRNA |
| CMV/EF1 hybrid promoter | Allows efficient, high-level expression of your recombinant protein |
| *Eco*RV and *Pac*I restriction enzyme sites | Entry points for gene insertion behind the CMV/EF1 promoter (see page 18 for sequencing primers used for analyzing CMV/EF1 promoter-gene of interest junction) |
| Simian virus 40 (SV40) polyadenylation signal | Allows efficient transcription termination and polyadenylation of mRNA |
| *Sfi*I restriction enzyme sites | Allows for removal of the CMV/EF1 expression cassette when using vector for single-subunit expression |
| Dihydrofolate reductase (DHFR) gene | Allows selection of transfected CHO-S™ cells using methotrexate (MTX) (Kaufman *et al.*, 1985) |
| Puromycin resistance gene (*pac*) (Puromycin N-acetyl-transferase) | Allows selection of transfected CHO-S™ cells using Puromycin (de la Luna & Ortin, 1992; Lacalle *et al.*, 1989; Vara *et al.*, 1985) |
| pMB1 origin of replication | Allows high-copy number replication and growth in *E. coli* (Lin-Chao *et al.*, 1992; Yanisch-Perron *et al.*, 1985) |
| Kanamycin resistance gene (*aph*) (aminoglycoside phosphotransferase, also known as kanamycin kinase type I) | Allows selection of transformants in *E. coli* (Oka *et al.*, 1981; Vakulenko *et al.*, 1987) |

## Unique restriction enzyme recognition sites

The Freedom™ pCHO 1.0 vector contains the following unique restriction enzyme recognition sites.

| Position of cut | Restriction enzyme | Recognition site |
|---|---|---|
| 1 | NdeI | CATATG |
| 540 | Sse8647I | AGGWCCT |
| 830 | ScaI | AGTACT |
| 2,134 | BplI | GAGNNNNNCTC |
| 2,738 | BaeI | ACNNNNGTAYC |
| 3,009 | BamHI | GGATCC |
| 3,521 | BclI | TGATCA |
| 3,597 | NheI | GCTAGC |
| 4,258 | SgrAI | CRCCGGYG |
| 4,258 | Sse232I | CGCCGGCG |
| 5,737 | AloI | GAACNNNNNNTCC |
| 5,765 | AvrII | CCTAGG |
| 5,776 | BstZ17I | GTATAC |
| 6,053 | EcoRI | GAATTC |
| 6,942 | FalI | AAGNNNNNCTT |
| 7,082 | FseI | GGCCGGCC |
| 7,834 | EcoRV | GATATC |
| 7,846 | PacI | TTAATTAA |
| 8,397 | FspI | TGCGCA |
| 8,701 | AscI | GGCGCGCC |
| 8,917 | BsiWI | CGTACG |
| 8,977 | RsrII | CGGWCCG |
| 9,829 | KpnI | GGTACC |
| 10,240 | PmlI | CACGTG |
| 12,070 | PvuI | CGATCG |
| 12,070 | AsiSI | GCGATCGC |
| 12,143 | SspI | AATATT |
| 12,411 | NruI | TCGCGA |

## Non-cutting restriction enzymes

The Freedom™ pCHO 1.0 vector lacks recognition sites for the following restriction enzymes: *Acl*I, *Fsp*AI, *Pme*I, *Psr*I, *Sbf*I, *Srf*I, *Swa*I.

# Appendix E

## Accessory products

### Freedom™ CHO-S™ Kit products

Many of the components supplied with the Freedom™ CHO-S™ Kit are also available separately. For more information, go to **www.thermofisher.com/lifescience** or contact Technical Support (see page 68).

| Item | Amount | Catalog no. |
|---|---|---|
| CHO-S™ Cells (cGMP-banked) and Media Kit (includes 1 × $10^7$ CHO-S™ Cells, CD CHO Medium, and L-glutamine) | 1 kit | A1155701 |
| One Shot™ TOP10 Chemically Competent *E. coli* | 10 reactions<br>20 reactions | C4040-10<br>C4040-03 |
| FreeStyle™ MAX Reagent | 1 mL | 16447-100 |
| OptiPRO™ SFM | 100 mL<br>1000 mL | 12309-050<br>12309-019 |
| L-glutamine, 200 mM, liquid | 100 mL | A2916801 |
| CD FortiCHO™ Medium | 1000 mL | A1148301 |
| Anti-Clumping Agent | 20 mL<br>100 mL | 0010057AE<br>0010057DG |
| Puromycin, liquid | 20 mL<br>10 × 1 mL | A1113802<br>A1113803 |

### Gibco™ custom media & Gibco™ services

Through our Gibco™ custom media capability and Gibco™ services, we can develop cloning or growth media formulations specifically suited to your cells. We can provide the best nutrient media delivery scheme for your recombinant cell line, optimizing a Gibco™ medium or one in the public domain, or your own formulation. All final media manufacturing is performed in our ISO-9001 certified, QSR/cGMP-compliant facilities and held to the same high standards as our own Gibco™ catalog products, ensuring scalability, robustness, and compliance. For more information, go to or **www.thermofisher.com/lifescience** contact Technical Support (see page 68).

### ProBioGen AG cell line development & manufacturing services

For more information of ProBioGen AG's cell line development services, including manufacturing, go to **www.probiogen.de.**

## Additional products

The products listed below may be used with the Freedom™ CHO-S™ Kit. For more information, go to **www.thermofisher.com/lifescience** or contact Technical Support (see page 68).

| Item | Amount | Catalog no. |
|---|---|---|
| Trypan Blue Stain | 100 mL | 15250-061 |
| PureLink™ HiPure Plasmid Midiprep Kit | 25 preps | K2100-04 |
| FoamAway™ Irradiated AOF | 500 mL in a 1000 mL bag | A10369-02 |
| Dynamis™ Medium (liquid) | 1000 mL | A2661501 |
| Dynamis™ AGT™ Medium | 1L<br>10 L<br>100 L<br>10 Kg | A2617504<br>A2617501<br>A2617502<br>A2617503 |
| EfficientFeed™ C+ AGT™ Supplement | 1 L<br>10 L<br>100 L | A25031-04<br>A25031-05<br>A25031-01 |
| Neon™ Transfection System | 1 each | MPK5000 |
| Neon™ Transfection System, 100 µL Kit | 50 reactions<br>192 reactions | MPK10025<br>MPK10096 |

# Appendix F

## Safety

**WARNING! GENERAL SAFETY.** Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the **Documentation and support** section in this document.

# Chemical safety

**WARNING! GENERAL CHEMICAL HANDLING.** To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the **Documentation and support** section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

## Biological hazard safety

**WARNING! BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at: **www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf**
- World Health Organization, Laboratory Biosafety Manual, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at: **www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf**

# Documentation and support

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